Interplay between Glucocerebrosidase 1 and Glucocerebrosidase 2; potential implications for the pathogenesis of Gaucher and Parkinson's diseases.

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I, Derek Gerard Burke confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Gaucher disease (GD) is a lysosomal storage disorder with wide clinical heterogeneity, caused by mutations in the *GBA* gene leading to low or deficient levels of the lysosomal enzyme Glucocerebrosidase (GBA1). Mutations in GBA1 are also the single most common genetic risk factor for developing Parkinson's disease (PD). However, the majority of GD patients and carriers do not develop PD, suggesting the possibility of other disease modifying factors. GBA2 is a non-lysosomal enzyme capable of hydrolysing the same substrates as GBA1 and hence may be a potential disease modifying factor.

Initially, assays for GBA1 and GBA2 were developed to record the relative activities in a range of tissue types. Marked tissue differences in GBA1 and GBA2 activities were recorded with GBA2 the predominant enzyme in brain. Assessment of GBA1 and GBA2 in GD, PD and dystonia patient leucocytes revealed, as expected, decreased GBA1 activity in GD samples. However, 13% of idiopathic PD and dystonia patients also displayed decreased activity. Leucocyte GBA2 activity was found to be elevated in half of GD patients and in brain from a GD mouse model. One patient with mild GD but profound PD had undetectable GBA2 activity.

Cellular models of GBA1 and/or GBA2 inhibition, oxidative stress, and mitochondrial dysfunction were used to further study the interplay between GBA1 and GBA2 using SH-SY5Y cells. Inhibition of GBA1 was not found to be associated with an increase in GBA2 activity or protein expression. Similarly, inhibition of GBA2 was not found to be associated with changes in GBA1 activity. Loss of mitochondrial function or oxidative stress was not found to cause loss of GBA1 activity while GBA1 inhibition did not increase cellular susceptibility to oxidative stress. Measurement of ceramides using mass spectrometry did not reveal any downstream effects of GBA1 or GBA2 inhibition on ceramide levels.

Table of Contents

List of figures

List of Tables

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List of abbreviations

3OMD	3-O-Methyldopa
4MU	4-Methylumbelliferone
5-HIAA	5-hydroxyindoleacetic acid
AAO	Age at onset
ACE	Angiotensin-converting enzyme
AD	Alzheimer disease
AMP	dNM - N-(5-adamantane-1-yl-methoxy-pentyl)-Deoxynojirimycin
AMRF	Action myoclonus-renal failure syndrome
ANOVA	Analysis of variance
ARCA	Autosomal recessive cerebellar ataxia
BCA	Bicinchoninic acid
B-GAL	Beta-galactosidase
BMT	Bone marrow transplantation
BSA	Bovine serum albumin
CBD	Corticobasal degeneration
CBE	Conduritol B-epoxide
CCL18	CC chemokine ligand 18
CMA	Chaperone-mediated autophagy
CNS	Central nervous system
CoQ10	Coenzyme Q10

CS	Citrate synthase
CSF	Cerebrospinal fluid
DLB	Dementia with Lewy bodies
DMEM/F12	Dulbecco's Modified Eagle's Medium/Ham's F-12 nutrient mixture
DMS0	Dimethyl sulfoxide
DNTB	5,5'-Dithio-bis (nitrobenzoic acid)
DTNB	[5, 5` dithio-bis (2-nitrobenzoic acid)]:
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ERAD	Endoplasmic reticulum associated degradation
ERT	Enzyme replacement therapy
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBA1	Lysosomal glucocerebrosidase (beta-glucosidase)
GBA2	Non-lysosomal glucocerebrosidase (beta-glucosidase)
GCS	Glucosylceramide synthase
GD	Gaucher disease
GlcCer	Glucosylceramide (Glucocerebroside)
GlcCerT	Ceramide glucosyl-transferase
GlcSph	Glucosylsphingosine

GSH	Glutathione
GSL	Glycosphingolipid(s)
GWAS	Genome-wide association study
H2O2	Hydrogen peroxide
HBSS	Hanks' Balanced Salt Solution
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HSA	Human Serum Albumin
HSCT	Haematopoietic stem cells transplantation
HSP	Hereditary spastic paraplegia
HVA	Homovanillic acid
iPD	Idiopathic Parkinson's disease
l-DOPA	l-3,4-dihydroxyphenylalanine
LDS	Lithium dodecyl sulphate
LIMP2	Lysosomal integral membrane protein type 2
LRRK	Leucine-Rich Repeat Kinase 2
LSD	Lysosomal storage disease
M6PR	Mannose-6-phosphate receptor
MOPS	3-(N-morpholino)propanesulfonic acid
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

MRM	Multiple reaction monitoring
MS	Mass spectrometry
MSA	Multiple system atrophy
MTT	3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
MV	McIlvaine (buffer)
NaT	Sodium taurocholate
NBDNJ	N-butyldeoxynojrimicin
NBIA	Neurodegeneration with brain iron accumulation
nGD	Neuronopathic Gaucher disease
PBS	Phosphate buffered saline
PD	Parkinson’s disease
PDD	Parkinson’s disease with dementia
PD-GBA1	Parkinson's disease patients with GBA1 mutation(s)
PSP	Progressive supranuclear palsy
PVDF	Polyvinyl difluoride
QSBB	Queen Square Brain Bank
ROS	Reactive oxygen species
RT	Room temperature
SCARB2	Scavenger receptor class B member 2 gene
SN	Substantia Nigra
SNP	Single nucleotide polymorphism

SNpc	Substantia Nigra pars compacta
SRT	Substrate reduction therapy
TBST	Tris-buffered saline tween-20
T-HEX	Total beta-hexosaminidase
TRAP	Tartrate-resistant acid phosphatase
UPDRS	Unified Parkinson's Disease Rating Scale
UPLC-MS/MS	Ultra Performance Liquid Chromatography tandem mass spectrometry
UPR	Unfolded protein response
UPS	Ubiquitin–proteasome system

Chapter 1 Introduction

1.1 Lysosomes

Lysosomes were first described by the Belgian biochemist Christian de Duve in the 1950s [1] . The name lysosome derives from the Greek words lysis, to separate, and soma, body [2]. Lysosomes are small, membrane-bound organelles found in all eukaryotic cells. They have a number of functions including digesting ingested material, molecular turnover, autophagy, receptor recycling, cell death as well as extracellular roles. They contain a range of water soluble hydrolases which are maximally active in the acidic environment (pH4-5) of the lysosome. The acidic environment is maintained by ATP dependent proton pumps. There are more than 60 lysosomal enzymes which together can break down proteins, polysaccharides, nucleic acids, lipids, organelles, bacteria and particles. Non-enzymatic proteins and cofactors such as the saposins enable the water soluble enzymes to act on lipids [3].

1.2 Lysosomal storage disorders

The concept of lysosomal storage disease (LSD) was established by H.G Hers in 1963 who identified the deficiency of lysosomal alpha-glucosidase in glycogen storage disease II (Pompe disease) [4]. LSDs can arise due to lysosomal enzyme deficiencies, defective trafficking defect of lysosomal enzymes, defects in soluble non-enzymatic lysosomal proteins and defects in lysosomal membrane proteins [5]. Enzyme deficiency will usually cause the accumulation of the incompletely catabolised substrate within the lysosome leading to progressive impairment of the affected cell and subsequently the organ or cell system. More than 45 disorders have been described so far affecting the degradation of a number of substrates including glycoproteins, glycolipids, mucopolysaccharides and oligosaccharides. Individually they are rare but collectively they have an incidence of 1 in 5000 live births and the incidence may even be higher with many cases being un- or misdiagnosed [6]. The LSDs apart from the x-linked Hunter disease, Fabry disease and LAMP2 deficiency are autosomal recessive disorders [7]. They are usually classified according to the

type of substrate that accumulates e.g. mucopolysaccharidoses, lipidoses, sphingolipidoses, oligosaccharidoses (glycoproteinoses), mucolipidosis etc. [8].

1.3 Sphingolipidoses

Gaucher disease, the subject of this thesis belongs to a group of lysosomal storage disorders known as the sphingolipidoses i.e. disorders of lysosomal sphingolipid metabolism. All eukaryotic cells are surrounded by a cell membrane composed of a lipid bilayer. The three main classes of lipids in these membranes are sphingolipids, glycerolipids and sterols [9]. Sphingolipids are amphipathic molecules that play an important role the structure and fluidity of the membrane and in signal transduction [10]. The backbone of all sphingolipids is a sphingoid long chain base, usually sphingosine or sphinganine, to which a fatty acid is attached by an amide bond [11]. Sphingosine differs from sphinganine in having a *trans* 4-5 double bond [9]. The simplest sphingolipid is ceramide which consists of a fatty acid residue attached to a sphingosine backbone at C-2 by an amide link [12]. Ceramide can receive a phosphocholine headgroup to form sphingomyelin, be phosphorylated to ceramide 1-phosphate, or be glycosylated by glucosylceramide synthase or galactosylceramide synthase to generate the cerebrosides glucosylceramide (GlcCer) or galactosylceramide [13]. The synthesis of glucosylceramide by glucosylceramide synthase occurs on the cytosolic leaflet of the Golgi apparatus [14]. Cerebrosides are the building block for glycosphingolipids (GSLs). Glycosphingolipid biosynthesis occurs in a stepwise fashion, with an individual sugar added first to ceramide and then subsequent sugars transferred by glycosyltransferases from nucleotide sugar donors [15]. Ninety percent of mammalian GSLs are based on GlcCer with the remainder based on galactosylceramide [16]. Sulphated cerebrosides are sulphatides. The addition of more than one sugar residue forms globosides while three or more sugar residues including a sialic acid forms gangliosides [15]. While more than 500 different carbohydrate structures have been described in GSLs, the main sugars are glucose, galactose, fucose, N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) and sialic acid (N-acetylneuraminic acid) [9]. GSLs have many functions including

senescence, apoptosis, cell proliferation, inflammation, endocytosis, intracellular transport, and cell migration [17].

Sphingolipidoses are a group of lysosomal storage diseases characterised by the accumulation of sphingolipids [18]. They are primarily caused by inherited defects in genes encoding the lysosomal enzymes (or their non-enzymatic cofactors) required for sphingolipid catabolism in late endosomes/lysosomes but some are due to defects in lysosomal membrane proteins [19]. Examples of enzyme defects include GM1 and GM2 gangliosidoses, metachromatic and Krabbe leucodystrophys and Gaucher and Fabry diseases (**Figure 1.1**) [20].

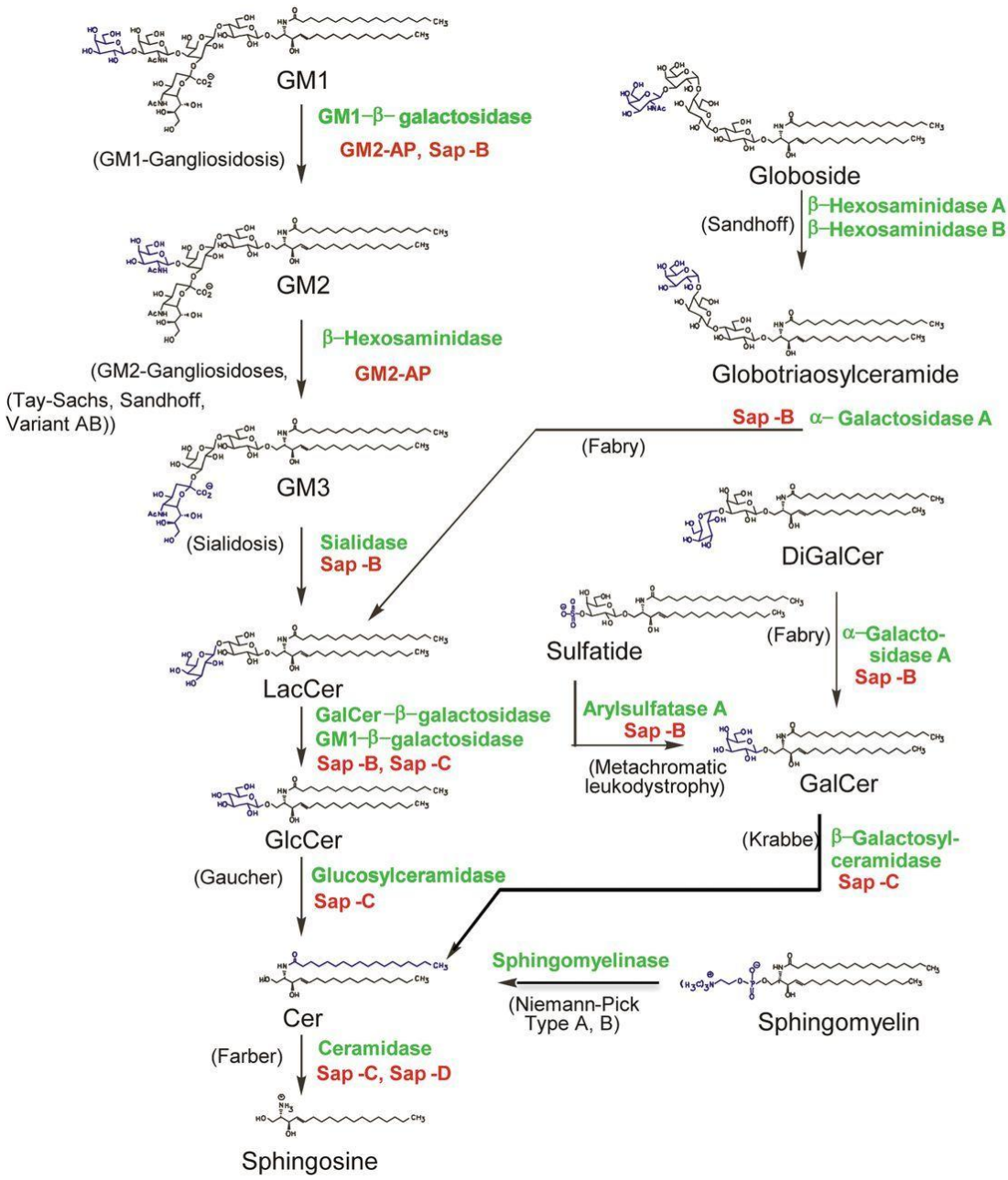

Figure 1.1 - **Pathway of lysosomal sphingolipid degradation.**
The known metabolic diseases in brackets, the responsible enzymes in green and those of SAPs necessary for *in vivo* degradation in red are indicated. Source: Metabolic and cellular bases of Sphingolipidoses. Konrad Sandhoff. Biochemical Society Transactions Dec 2013 [21].

1.4 Gaucher disease

1.4.1 Introduction

Gaucher disease (GD) was first described in 1882 by Phillipe Gaucher, a French medical student in his doctoral thesis [22]. It is a lysosomal storage disorder (LSD) caused by a mutation in GBA1 leading to low or deficient levels of the lysosomal β-glucocerebrosidase (β-glucosidase/β–glucosylceramidase) (EC 3.2.1.45). Lysosomal β-glucocerebrosidase (herein referred to as GBA1) is responsible for the penultimate step of the lysosomal degradation of glycosphingolipids i.e. the degradation of glucosylceramide (also known as glucocerebroside) to ceramide and glucose. The ceramide is further degraded by ceramidase to sphingosine and fatty acid. An alternative substrate, glucosylsphingosine, is also degraded by GBA1 into glucose and sphingosine (**Figure 1.2**) [23].

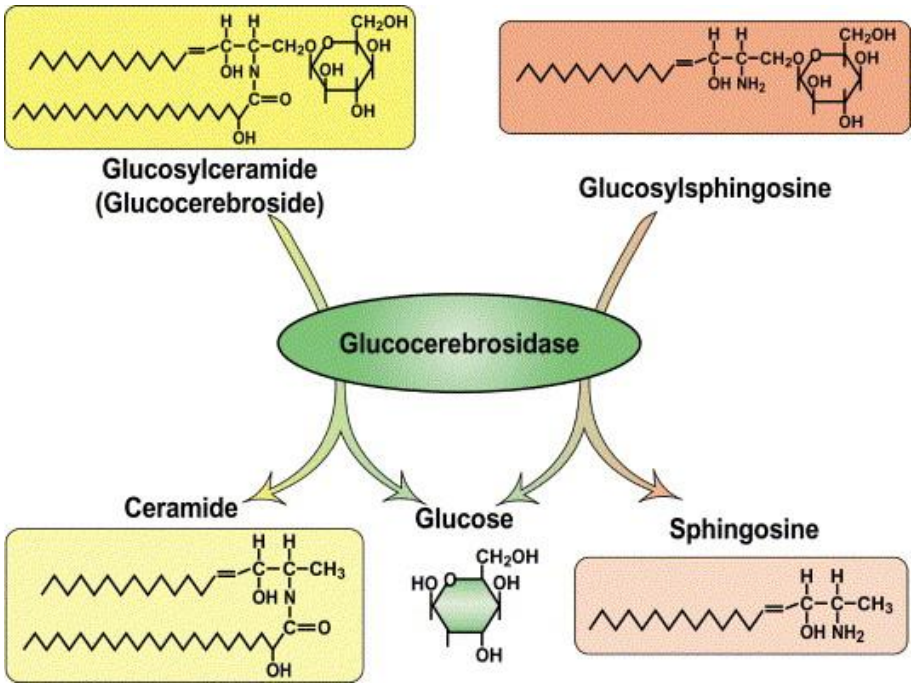

Figure 1.2 - GBA1 (glucocerebrosidase) metabolism of glucosylceramide and glucosylsphingosine (from Sidransky *et al.*[23])

1.4.2 GBA1

GBA1 is composed of 497 amino acids and is present in the lysosomes of all tissues, which explains the multi-organ nature of the disease [24]. The main source of

glucosylceramide (GlcCer) is from the membranes of blood cells, the turnover of which takes place in the lysosomes of macrophages [25].

In patients with Gaucher disease, the breakdown of GlcCer is insufficient, leading to the accumulation of large quantities of the substrate within the lysosomes of macrophages, especially in tissues of the reticuloendothelial system. Such cells are referred to as Gaucher cells [22]. It is not clear how GlcCer itself or the consequent imbalances of ceramide, sphingosine, and sphingosine 1-phosphate affects Gaucher disease. it is also unclear how GlcCer accumulation in lysosomes leads to cellular pathology, and whether GlcCer can escape the lysosomes and interact with different cellular and biochemical pathways in other organelles [26]. GBA1 is important for the production of ceramide from GlcCer, in what is known as the salvage pathway. This is the most energy efficient method of synthesising ceramide in post-mitotic cells [27].

Unlike other lysosomal hydrolases, which are targeted to lysosomes through binding to the mannose-6-phosphate receptor (M6PR), GBA1 is targeted to lysosomes through an M6PR-independent mechanism; i.e. binding to the lysosomal integral transmembrane protein type 2 (LIMP-2). The binding between GBA1 and LIMP-2 enables these two proteins to be reciprocally associated in the endoplasmic reticulum and trafficked continuously to the lysosomes [28].

1.4.3 Clinical presentation

GD is the most common of the LSDs with an overall incidence of 1:40,000 to 1:100,000 individuals, but it is much more common amongst individuals of Ashkenazi Jewish origin [29, 30]. GD is usually delineated into three types. Type 1 is by far the most common and has historically been distinguished from types 2 and 3 by the lack of primary central nervous system involvement. Type 1 GD has variable presentation and course of disease with wide clinical heterogeneity. It typically manifests with visceral, haematological and skeletal symptoms such as splenomegaly, anaemia and acute bones crises [22]. However, although usually classified as non-neuronopathic, neurological disease has been diagnosed in GD type I patients but the neurological signs and symptoms are of a totally different

kind from and, in the majority of cases, of much less severity than those associated with types II and III GD disease [31]. Type 2 is the acute neuronopathic form of Gaucher disease (nGD) which presents in infancy and is rapidly progressive and fatal within 1-2 years. Type 3 is the chronic Neuronopathic form with a severity between that of type II and III [22]. There is also a rare perinatal-lethal Gaucher disease phenotype which features hydrops fetalis, *in utero* fetal death and neonatal distress. When hydrops is absent, neurological involvement begins in the first week and leads to death within three months [32].

1.4.4 Pathophysiology

Gaucher cells are lipid laden macrophages which have a "crumpled paper" appearance with eccentric nuclei under light microscopy as seen in **Figure 1.3a** [33]. This appearance is caused by the presence of GlcCer which aggregates in a characteristic twisted fibrillar arrangement that can be observed by electron microscopy as seen in **Figure 1.3b** [34]. Gaucher cells mainly infiltrate bone marrow and the spleen but also infiltrate the liver and other organs and are thought to be a major cause of manifestations of the disease [35]. While GlcCer and glucosylsphingosine accumulation and subsequent proliferation of lipid laden macrophages account for the visceral manifestations of GD, the precise mechanism particularly of non-visceral symptoms is still uncertain. One proposed mechanism is the activation of pro- and anti-inflammatory pathways by incorrectly folded proteins in the endoplasmic reticulum [36]. Analysis of the lipid composition of liver, spleen, brain, cerebellum and cerebrospinal fluid from a GD type II patient who died at the age of 5 months demonstrated a marked increase of total glycolipids not only in the peripheral tissues but also in the brain cerebellum and cerebrospinal fluid, with a prevalence of GlcCer. Interestingly, the fatty acid composition of GlcCer showed a prevalence of stearic acid in the central nervous system, while in the peripheral tissues palmitic acid was prevalent. This result suggests a different origin of the GlcCer stored in different tissues [37].

1.4.5 Laboratory findings and diagnosis

Due to the phenotypic diversity it can often take some time before a diagnosis of GD is suspected and confirmed. Laboratory findings include thrombocytopenia, anaemia, leucopoenia and increased erythrocyte sedimentation rates. Bone marrow may have the characteristic Gaucher cells [35] shown in Figure 1.3.

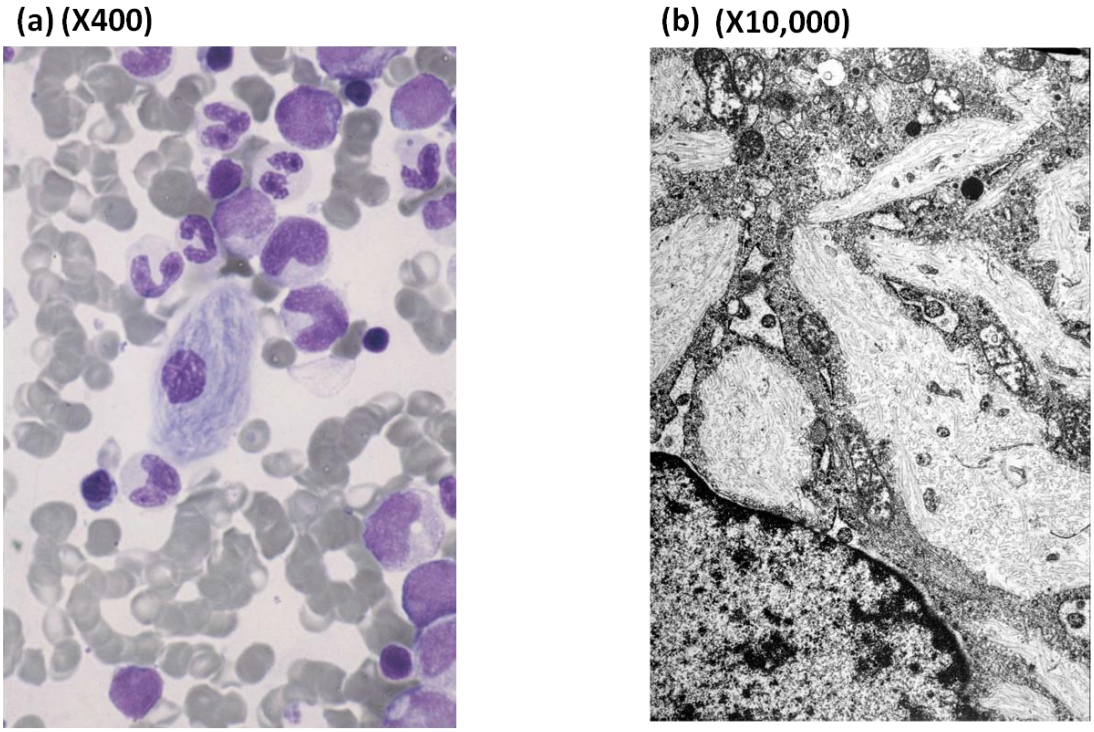

Figure 1.3- Microphotographs of Gaucher cells.

(a) 1.Bone marrow aspirate with stripy, basophilic, storage cell with the classic "crumpled paper" appearance. MGG stain (X400); (b) EM showing part of a nucleus and several 'angulate lysosomes' with tubular storage inclusions (X10,000). Pictures courtesy of Glenn Anderson, Histopathology, Great Ormond Street Hospital.

Biochemical findings may include increased tartrate resistant acid phosphatase (TRAP), angiotensin converting enzyme (ACE), liver enzymes and urea. The lysosomal enzyme b-hexosaminidase is sometimes elevated [38] and it has been suggested that it could be used as a screening test [39], however marked variation in levels makes it unreliable as a screening test [40]. See Chapter 5 for further discussion of total beta-hexosaminidase in GD and oxidative stress.

Chitotriosidase, a chitinase is hypersecreted by the affected macrophages, and as shown in **Table 1.1**, plasma levels are markedly elevated in GD patients compared to unaffected individuals and other lysosomal storage disorders [41]. Plasma chitotriosidase activity levels correlate with liver and spleen volume, haemoglobin concentration, platelet count, and bone manifestations [42]. However, about 5% of the general population have a null allele in their chitotriosidase gene and it is therefore not of use in those GD patients who are unable to produce it [41].

Table 1.1 Chitotriosidase activities in plasma from controls, Gaucher disease patients and patients with other lysosomal storage diseases.

Condition	Plasma Chitotriosidase (nmol / hr / mL)
Unaffected	0 - 150
Gaucher	2,000 - 35,000
Niemann Pick	178 – 3372
GM1 gangliosidosis	174 – 1984
Wolman, CESD	284 – 1015
Krabbe leucodystrophy	53 – 1589

An alternative macrophage biomarker is CC chemokine ligand 18 (CCL18), originally named pulmonary and activation-regulated chemokine (PARC). Plasma CCL18 originates from Gaucher cells and levels reflect the overall body burden of Gaucher cells. Plasma CCL18 levels are elevated 10- to 50-fold above normal values in patients with active GD [43].

The diagnosis of GD is confirmed by demonstrating deficient GBA1 activity in patient samples with subsequent mutation analysis. GBA1 activity is usually assayed in dried blood spots or leucocytes, but cultured fibroblasts may be used [22].

1.4.6 Treatment

Early treatment of Gaucher disease involved splenectomy to ameliorate the consequences of hypersplenism. Haematological parameters returned to normal in all cases and remained so throughout the follow-up period [44]. Splenectomy is now contraindicated in all but emergency situations such as splenic rupture or profound thrombocytopenia necessitating platelet transfusions or life-threatening internal haemorrhage. In such instances, partial, rather than total, splenectomy should be considered [45].

The first specific treatment for GD was bone marrow transplantation (BMT) or haematopoietic stem cells transplantation (HSCT) for severe type I and 3 patients [46]. HCT is effective in alleviating most disease manifestations of GD including arresting further neuropsychological deterioration in type III disease and greatly reducing skeletal problems in severe early onset type I disease [47]. Two years after BMT, plasma chitotriosidase activity in eight patients had fallen by over 93% and continued to fall until 6 patients had normal levels and 2 patients had activities slightly above the reference range [48]. However BMT and HSCT is associated with significant morbidity and mortality [47] and these treatments are no longer offered due to the availability of new therapies with greater benefit to risk ratios [35].

Shortly after the discovery of the enzymatic deficiency in GD [49, 50], it was suggested that replacing or supplementing the deficiency by exogenous administration of the enzyme could be a potential therapeutic approach [51]. Initial experiments using purified GBA1 isolated from human placenta showed promise [52] but it wasn't until improved large-scale purification techniques were developed that larger trials could take place [53]. Outcomes improved after it was found that the enzyme could be targeted to macrophages by the sequential enzymatic removal of N-acetylneuraminic acid, galactose and N-acetylglucosamine with exoglycosidase to expose mannose residues which bind to mannose lectin on the macrophage cell surface [54].

This led to the development by Genzyme, of Ceredase (alglucerase), a purified macrophage targeted GBA1 extracted from human placenta as an enzyme

replacement therapy (ERT) for GD. This was the first commercial enzyme replacement therapy for any lysosomal storage disorder [55]. To obtain sufficient protein to treat an adult GD patient with Ceredase required several hundred metric tons of placentas each year and at the peak period of manufacturing, placentas from about one-third of the world's births found their way to the tissue banks of the Pasteur Mérieux of Lyon, France. There, human immunoglobulin was extracted from the tissue, after which the residue was obtained by Genzyme for enzyme extraction, purification and remodelling to produce Ceredase [55].

Subsequently in 1995, Genzyme launched Cerezyme (imiglucerase), a recombinant human glucocerebrosidase expressed in genetically engineered Chinese hamster ovary cells. As with the purified placental product, this enzyme also required further modification by exoglycosidases to expose glycan residues which mediate delivery macrophages [56]. A study of data from the Gaucher Registry of 1028 GD type 1 patients after 2-5 years enzyme replacement therapy with either Ceredase or Cerezyme demonstrated that ERT prevents progressive manifestations of, and ameliorates GD–associated anaemia, thrombocytopenia, organomegaly, bone pain, and bone crises. However, some of the more severely affected patients continued to have signs or symptoms of the disease, and therapy was more effective when initiated before irreversible damage occurs [57].

Other ERTs for GD have now been developed including VPRIV® (velaglucerase) produced using human fibroblasts by Shire [58] and ELELYSO® (taliglucerase alfa) produced using carrot cells by Pfizer and Protalix [59]. All of the ERT therapies have similar therapeutic benefits and effectiveness on normalising haematological parameters [60]. ERT is not a cure, and requires regular intravenous administration of the drug for the life time of the patient. The dose and frequency of administration varies from country and may be individualised for each patient [61, 62]. ERT is not effective at treating the neurological manifestations of GD type 2 or 3 as it does not cross the blood-brain barrier [63].

An alternative approach to treatment proposed in 1980 is to reduce the amount of substrate accumulating by inhibiting its synthesis [64]. Substrate reduction therapy

(SRT) uses small molecules to slow the rate of glycolipid biosynthesis which should lead to fewer GSLs entering the lysosome thereby reducing the rate of storage. Complete balance might be achieved in patients with residual GBA1 activity [65]. Advantages of using such an approach is that the treatment would be oral, avoiding regular intravenous infusion, and would use small molecules which would be less likely to cause an immune response compared to ERT, and which unlike ERT could cross the blood-brain barrier potentially treating neurological manifestations [65]. The first compound approved for SRT in GD was N-butyldeoxynojirimycin (NBDNJ) marketed as ZAVESCA® (Miglustat) which is a glucose analogue which acts as an orally active competitive, reversible inhibitor of glucosylceramide synthase [66].

In the US, Zavesca is indicated as monotherapy for the treatment of adult patients with mild to moderate GD1 for whom ERT is not a therapeutic option (e.g. due to allergy, hypersensitivity, or poor venous access). In the European Union, Zavesca is also indicated for the treatment of progressive neurological manifestations in adult patients and paediatric patients with Niemann-Pick type C (NP-C) disease [67]. It can produce side effects including diarrhoea, weight loss, hand tremors and possible peripheral neuropathy although these generally regress with dose reduction or treatment discontinuation [35]. Gastrointestinal events, mainly diarrhoea, have been observed in more than 80% of patients, either at the outset of treatment or intermittently during treatment. The mechanism is most likely inhibition of intestinal disaccharidases such as sucrase-isomaltase in the gastrointestinal tract leading to reduced absorption of dietary disaccharides [67]. Approximately 37% of patients in clinical trials in type 1 Gaucher disease, and 58% of patients in a clinical trial in Niemann-Pick type C disease reported tremor on treatment [67]. Despite the fact that it crosses the blood–brain barrier, Miglustat has not been found to have any effect on neurological symptoms in GD3 [35]. It has been also found that Miglustat treatment in addition to ERT does not appear to have significant benefits on the neurological manifestations of GD3 but may have positive effects on systemic disease (pulmonary function and chitotriosidase activity) in patients with GD3 [68].

Another substrate inhibitor, Eliglustat (Cerdelga®, Sanofi-Genzyme) was granted a marketing authorization in 2015. It too is an orally administered GlcCer synthase inhibitor, but is an analogue of the ceramide part of glucosylceramide and is therefore a more specific and more potent inhibitor than Miglustat [35]. In the phase 3 Study (ENCORE) of Eliglustat Tartrate (Genz-112638) in patients with GD who had reached therapeutic goals with ERT at 1 year, Eliglustat was found to be as effective as imiglucerase enzyme therapy in maintaining stable platelet counts, haemoglobin concentrations, and spleen and liver volumes. Mean bone mineral density remained stable and was maintained in the healthy reference range throughout. When the study was extended, Eliglustat was well tolerated over 4 years and few patients withdrew because of adverse events that were considered related to the study drug. No new or long-term safety concerns were identified [69]. Eliglustat does not cross the blood-brain barrier and is therefore only suitable for non-neurological GD patients [70].

GD like many other lysosomal storage disorders is a good candidate for gene therapy as it is a monogenic disorder, only a small amount of enzyme is required to correct symptoms and cells have the ability to cross-correct i.e. the ability for extracellular LSD enzymes to be taken up and targeted to the lysosomes of otherwise enzyme-deficient cells [71]. Currently, lentiviral haemopoietic stem-cell gene therapy is being trialled in early-onset metachromatic leucodystrophy (MLD), a fatal demyelinating lysosomal disease with no approved treatment. Preliminary evidence has demonstrated the safety and therapeutic benefit of the treatment [72]. Gene therapy clinical trials are also underway for Fabry disease, Mucopolysaccharidosis type II (MPS II/Hunter syndrome), Mucopolysaccharidosis type III (MPS III/Sanfilippo syndrome) types A and B, infantile Batten's disease (INCL), and Pompe disease [71].

A preliminary gene transfer protocol was used on three adult GD3 patients using retroviral transduction of peripheral blood (PB) or bone marrow (BM) CD34+ cells with the G1Gc vector which uses the viral LTR promoter to express the human GBA1 cDNA. The corrected cells were then injected into patients. Results were disappointing as the GBA1 levels proved too low for any clinical effect [73].

Subsequently, lentiviral vector gene transfer techniques have been used in a murine disease model of type 1 GD with promising results. GBA1 activity above levels required for clearance of glucosylceramide from tissues resulted in reversal of splenomegaly, reduced Gaucher cell infiltration and restored haematological parameters [74]. There are currently a number of phase I trials of gene therapy for GD type 1 taking place [75] . Evaluation of AAV2/9 vector to transduce the nervous system and target gene expression to specific neural cell types following intravenous injection into fetal and neonatal mice, produced global delivery to the central (brain, spinal cord, and all layers of the retina) and peripheral (myenteric plexus and innervating nerves) nervous system and may be a potential system for treating neuronopathic GD [76].

Loss-of-function diseases such as GD are often caused by missense mutations that disrupt the three-dimensional conformation of mutant proteins. Such misfolded proteins may be recognized by the quality control systems of the endoplasmic reticulum (ER) and degraded, retained in the ER, or abnormally glycosylated and mis-trafficked. Pharmacological chaperone therapy is based on the concept of using small-molecule ligands or pharmacological chaperones that can interact with mutant proteins, enhancing their stability, and allowing for correct trafficking. As a result, the enzymatic activity of the mutant protein is partially rescued [75]. Isofagomine, is an active site inhibitor of GBA1 that acts as a pharmacological chaperone and has shown promise in mouse studies with increases in GBA1 enzyme activity and protein levels and a decrease in accumulated GlcCer and glucosylsphingosine of 75 and 33%, respectively [77]. In phase II clinical trials it was shown that isofagomine (Plicera®), increased patient GBA1 activity without unwanted side effects, however, the effects were not sufficient to significantly reduce symptoms of GD [78]. Enhancement of GBA1 activity was demonstrated using α-1-C-tridecyl-DAB at an effective concentration 10-times lower than isofagomine. α-1-C-Tridecyl-DAB is the first example of a pyrrolidine iminosugar as a new class of pharmacological chaperones with the potential for treatment of GD [78]. Ambroxol is a licensed expectorant, which has been demonstrated to increase GBA1 activity and protein levels and therefore be a potential chaperone therapy in

GD. An open label pilot study of Ambroxol in conjunction with ERT in patients with nGD demonstrated its safety, tolerability and effectiveness. It was shown that it significantly increased lymphocyte GBA1 activity, permeated the blood–brain barrier, and decreased glucosylsphingosine levels in the cerebrospinal fluid. Myoclonus and seizures improved in all patients. Relief from myoclonus led to impressive recovery of gross motor function in two patients, allowing them to walk again [79]. A pilot study in type I GD patients who were not receiving ERT also demonstrated tolerability and efficacy [80].

1.4.7 Monitoring of treatment

Monitoring of patients whether on treatment or not, includes laboratory testing of haematological parameters and biomarkers as well as regular clinical and radiological evaluations [35]. The levels of chitotriosidase tend to fall during successful treatment so it is a useful biomarker as well as screening/confirmatory test for the majority of GD patients who express it [41]. For those patients that express chitotriosidase it is a preferable marker to ACE and to acid phosphatase [81]. Tartrate-resistant acid phosphatase (TRAP) is neither specific for GD nor greatly elevated and the protein is unstable and is subject to wide analytical variability. ACE activity is subject to variable expression related to a common genetic polymorphism and is decreased by the use of frequently-prescribed ACE inhibitors [82].

A third of GD patients will be heterozygous for the chitotriosidase mutation and so while it is a useful marker for an individual patient, it has limited use in comparing patients [35]. Plasma CCL18 concentrations decrease during therapy, comparable to chitotriosidase and monitoring of plasma CCL18 levels is useful in monitoring disease progression and effectiveness of treatment, especially in patients who are deficient in chitotriosidase activity [83]. Glucosylsphingosine is elevated in GD and has been found to correlate with changes in chitotriosidase in the majority of GD patients on treatment who were informative for this marker [84].

1.4.8 Genetics

GD is an autosomal recessive disease. The gene encoding GBA1 is located on the long arm of chromosome 1 (1q21) and contains 11 exons. There is a highly homologous pseudogene (GBAP) at the same locus (16 kb downstream) which is responsible for recombination events between *GBAP* and *GBA1* e.g., RecNciI allele [35]. More than 400 mutations have been described in the *GBA1* gene [35]. Most of the disease alleles in GD are missense mutations that lead to GBA1 with decreased catalytic function and/or stability [85]. Several nonsense mutations have been described but occur in a heteroallelic state with a missense mutation. Other mutation types including gene fusions with the pseudogene, deletions, and frameshift mutations have been described. GD is a pan-ethnic disorder but is most common in the Ashkenazi Jewish population, where prevalence rates are estimated to be between 1:400-1:2500. Worldwide, birth prevalence rates of symptomatic GD have been estimated to be between 1:57,000 to 1:110,000 which translates into an overall prevalence rate of about 1:100,000 [6, 22, 30, 86]. The most common mutation in the Ashkenazi Jewish population is the N370S amino acid substitution which is associated with non-neuronopathic disease only. Many homozygotes for N370S do not come to medical attention until middle age or not at all. Other mutations such as the L444P are highly associated with Neuronopathic disease [22]. Six mutations account for about 96% of the mutant alleles among Ashkenazi Jews. Two of these, N370S and R496H, have been reported in mild non-neurological cases or in asymptomatic patients. Three others (84insG, L444P and IVS2+1G→A) are known to be involved in the rare severe neuropathic forms of GD. And V394L was reported in type 3 neuropathic form in combination with L444P or RecNciI allele [87]. The N370S mutation influences the flexibility of the loop 1 region of GBA1 resulting in enzyme with reduced catalytic activity with normal stability that is expressed at normal or near normal levels [88]. L444P is located in the hydrophobic core of the Ig-like domain. Mutations cause a local conformational change by disrupting the hydrophobic core, resulting in altered folding of this domain, producing unstable protein. This has been suggested to affect the interaction of the enzyme with saposin C [89].

1.4.9 Clinical heterogeneity

Studies of genotype-phenotype correlations reveal significant genotype heterogeneity among GD patients with similar clinical phenotypes. Conversely, individuals sharing the same genotype can present with and exhibit different disease phenotypes, clinical courses and responses to therapy [23]. For example, patients with oculomotor abnormalities have many different genotypes [90]. Differences have been observed among 24 sibling pairs in Canada with GD1 where there was concordance in 14 of the pairs but none between the other 10 pairs of siblings [91]. Subjects with genotype N370S/N370S can vary from asymptomatic adults to children with significant organomegaly, growth delay, or bone disease while a review of 35 patients with genotype L444P/L444P, each confirmed not to have a recombinant allele, demonstrated phenotypes ranging from death in early childhood, to autism to successful college students [92]. One of the few identified genotype–phenotype correlations involves mutation N370S which is encountered solely in patients with GD1. Since the observed frequency of N370S homozygotes is considerably less than would expected when calculated from the allele frequency in the Ashkenazi Jewish population, the majority of individuals with this genotype are probably asymptomatic or do not reach medical attention [93].

Mutation D409H is associated with an atypical GD phenotype, which includes calcification or fibrosis of the cardiac valves, corneal opacities, hydrocephalus, and dysmorphic features [94], However, not all individuals homozygous for D409H, develop this unique phenotype, and it is also associated with a type 2 phenotype [95].

Differences have been observed among monozygotic twins homozygous for the N370S allele who had cohabited all their lives where one twin suffered from fatigue, hepatosplenomegaly, thrombocytopenia and bone manifestations while the other twin remained asymptomatic [96]. Another group have described monozygotic twin sisters, born to consanguineous Moroccan parents, who are highly discordant for the manifestations of Gaucher disease. Both carry the N188S/N188S genotype. One twin has severe visceral involvement, epilepsy, and a

cerebellar syndrome, while her twin does not manifest any signs or symptoms of Gaucher disease but suffers from type 1 diabetes mellitus [97].

1.5 Action myoclonus-renal failure syndrome

LIMP2 encoded by the scavenger receptor class B member 2 gene (*SCARB2*), is a ubiquitously expressed transmembrane protein that is found predominantly in late endosomes and lysosomes and has been implicated in the biogenesis and maintenance of endosomes and lysosomes [98, 99]. LIMP2 mediates the mannose-6-phosphate independent trafficking of GBA1 to lysosomes [28]. Deficiency of LIMP2 causes action myoclonus-renal failure syndrome (AMRF) [100, 101]. AMRF is an autosomal recessive progressive myoclonus epilepsy associated with renal dysfunction that appears in the second or third decade of life. Cases with progressive myoclonus epilepsy associated with *SCARB2* mutations without renal compromise have also been reported. Additional neurological features can be demyelinating peripheral neuropathy, hearing loss and dementia. The course of the disease is relentlessly progressive [102]. LIMP2-deficient fibroblasts like those from patients with Gaucher disease (GD) show almost no active GBA1. However, white blood cells contain considerable amounts of residual enzyme. Consequently, AMRF patients do not acquire lipid-laden macrophages and do not show increased plasma levels of macrophage markers, such as chitotriosidase, seen in patients with GD. Plasma GlcCer concentrations were normal in the AMRF patients investigated as well as in LIMP2-deficient mice [103].

Saposin C deficiency, caused by a mutation in the PSAP gene is an extremely rare cause of GD [104]. So far, five unrelated patients have been reported, of which two displayed a GD3 phenotype and two displayed a GD1 phenotype [105].

1.6 GBA2 (EC3.2.1.45, GH116)

1.6.1 GBA2 Introduction

It been known since the role of GBA1 in GD was elucidated that there were other non lysosomal b-glucosidases in the cell which were not involved in GD including a cytosolic β-glucosidase present in various tissues but absent in fibroblasts and the lactase-phlorizin hydrolase exclusively present in the microvilli of the intestine [106]. Later, it was reported that human liver contains β-glucosidase activity active towards bile acid beta-glucosides, which was different from the previously described β-glucosidases [107]. Using conduritol β epoxide (CBE) which is an irreversible inhibitor of the lysosomal GBA1, It was subsequently confirmed that there was a CBE-insensitive non-lysosomal β-glucosidase which could metabolise glucosylceramide active in brain, spleen, fibroblasts and various cell lines and was not deficient in Gaucher disease [108]. The authors also demonstrated that the non-lysosomal enzyme behaved differently to GBA1 in the presence of sodium taurocholate (0.5% [mass/vol]) which stimulated the activity of the CBE-sensitive enzyme (GBA1) approximately two-fold whereas the CBE-insensitive enzyme activity was completely lost in the presence of sodium taurocholate. The presence in the assay of 1mg/mL of the GBA1 activator protein Saposin C resulted in a doubling in the activity of the CBE-sensitive enzyme while having no effect on the CBE-insensitive enzyme. The CBE-sensitive enzyme had a pH optimum of about 4.5 compared to 5.5 for the CBE-insensitive enzyme. While the physiological role of the CBE-insensitive enzyme was still unclear the authors suggested it was likely to be involved in non-lysosomal glycosphingolipid metabolism and questioned whether the enzyme may compensate for the GBA1 deficiency by metabolising some of the unhydrolysed substrate [108].

In 2006 Yildiz and colleagues generated a GBA2 deficient mouse model [109]. To their surprise they found despite being deficient in the bile acid b-glucosidase, the mice had normal plasma triglyceride, cholesterol and lipoprotein levels and normal bile acid compositions. The only clinical finding was that there was reduced fecundity in the male mice but no effects on female reproductive fitness were

observed. The infertility was associated with the accumulation of GlcCer in tissues especially the testes where GBA2 was highly expressed. Microscopic examination of sperm showed abnormally large, round heads (globozoospermia), abnormal acrosomes, and disordered mitochondria and defective mobility. GlcCer also accumulated in the brain and liver. They concluded that GBA2 was able to catalyse the hydrolysis of glucosylceramide as well as bile acid glucosides but did not play a major role in cholesterol and bile acid metabolism. Although there was an accumulation of GlcCer, there were no GD like symptoms in the affected mice. Yildiz suggested that two enzymes may not compensate for each other because of their unique tissue specific expression patterns and subcellular localizations. However the knock-out mice only lacked exons 5-10 and retained 50% of normal glucosidase activity [109].

Shortly afterwards, it was demonstrated that the previously described bile-acid (non-lysosomal) glucosylceramidase was in fact GBA2, which had been previously discounted as it been reported to be expressed specifically in liver and to be inactive against GlcCer [110].

More recently it has been shown that GBA1 is a second bile acid b-glucosidase. However while a deficiency of GBA1 lead to lysosomal GlcCer accumulation, and GBA2 to extra-lysosomal GlcCer accumulation, neither deficiency leads to an accumulation of bile acid β-glucoside [111].

The mouse GBA2 mRNA is most abundant in the testes and to a lesser extent in the brain with lesser amounts in other tissues, however the protein expression and activity levels are higher in the brain than in the testes [109]. The human GBA2 gene is on chromosome 9, mapping in position p.13.3. Human GBA2 mRNA is mainly expressed in the brain, heart, muscle, kidney and placenta and to a lesser extent in liver, spleen and lung. The protein contains 927 amino acids with a molecular weight of 104.6 kDa [112].

There is still debate about the localisation of GBA2 within the cell. Matern et al found GBA2 to be a single pass trans-membrane protein [112] while Boot and co-

workers found it to be located at or close to the cell surface. Yildiz et al stated that GBA2 is a resident ER protein [109]. They later generated GBA2-specific antibodies and developed an assay that discriminates between GBA1 and GBA2 without the use of detergent. They found that GBA2 is not an integral membrane protein but rather a cytosolic protein that tightly associates with cellular membranes localised at the ER and Golgi, which they suggest puts GBA2 in a key position for a lysosome-independent route of GlcCer-dependent signalling [113]. Others suggest that as GBA2 generated ceramide is rapidly converted to sphingomyelin, GBA2 must be in close contact with sphingomyelin synthase 1 (SMS1) which is located in the Golgi apparatus and SMS2 which is located on the cell surface [110, 111].

GBA2 was previously thought to be an intrinsic membrane protein with residues 689–708 predicted as a transmembrane domain but is easily extracted from cells in buffer without detergents. The previously predicted hypothetical transmembrane domain corresponds to H10, an internal helix in the catalytic domain of TxGH116 and the derived GBA2 model, which is incompatible with it being a transmembrane helix. A new model supports the peripheral membrane localization of GBA2, where it may bind to an intrinsic membrane protein or polar lipid head groups [114].

1.6.2 GBA2 clinically

It had been shown that administration of the GBA2 inhibitor, NBDNJ caused impairment of spermatogenesis in mice and it was suggested that it may have a role as a male contraceptive [115]. Later it was shown that GBA2 knockout mice have reduced male fecundity but are otherwise healthy [109]. NBDNJ marketed as Zavesca (Miglustat) is licensed as a substrate reduction therapy (see 1.4.6). Gaucher patients are given 100mg of NBDNJ, three times a day resulting in low micromolar plasma concentrations which should lead to complete GBA2 inhibition and following initiation of Miglustat therapy, Gaucher patients show even higher concentrations of GlcCer in erythrocytes than before therapy [116]. The growing long-term experience with Zavesca substrate reduction therapy indicates that this treatment is without major adverse effects but because of the evidence from mice, male patients seeking to conceive are advised to cease Zavesca treatment for three

months [117]. However, there was no evidence that Zavesca impaired spermatogenesis in healthy volunteers [118] which indicates that GlcCer metabolism is less important in humans than in mice. Zavesca (Miglustat) has been clinically licensed for over ten years for use in type 1 Gaucher disease, and does not give rise to ataxia or paraplegia [119].

Although GBA2 knock-out mice have no neurological symptoms it is now apparent that mutations in GBA2 can lead to neurological impairment in humans. It has been found that mutations in GBA2 cause hereditary spastic paraplegia (HSP) [120, 121], autosomal recessive cerebellar ataxia (ARCA)[122, 123] and a Marinesco-Sjogren-Like Syndrome [116].

Homozygosity mapping and whole-exome sequencing performed to identify the genetic origin of cerebellar ataxia in four unrelated consanguineous families of Tunisian descent identified mutations within the GBA2 gene. All affected individuals were homozygous for three different mutations in the GBA2 gene. The clinical presentation in all 10 patients (six male, four female) was progressive cerebellar ataxia which began in childhood or early adulthood. One affected individual also exhibited mild intellectual disability. The course of the disease was slowly progressive. There were no lipid abnormalities. One individual presented with hepatomegaly but other Gaucher disease features were absent in this and all the other patients. One individual had three children [122].

Using whole-exome sequencing, a Cypriot group identified a novel missense mutation (c.1780G > C [p.Asp594His] in the GBA2 gene in three patients from one family who presented with mixed features of cerebellar ataxia and spasticity. Spasticity was increased during the disease progression affecting initially the lower limbs and truncal muscles and later the upper limbs. Some additional features such as cognitive impairment, hearing loss, urinary incontinence and dysphagia often observed in other genetic diseases with spastic-ataxia as the predominant clinical feature were observed in this family as well [123].

Spastic paraplegia 46 (SPG46) refers to a locus mapped to chromosome 9 that accounts for a complicated autosomal-recessive form of hereditary spastic

paraplegia (HSP). Using next-generation sequencing in three independent families, four different mutations in GBA2 were identified (three truncating variants and one missense variant), which were found to cosegregate with the disease and were absent in controls. The overall phenotype was a complex HSP with mental impairment, cataract, and hypogonadism in males associated with various degrees of corpus callosum and cerebellar atrophy on brain imaging. The two affected men presented with bilateral testicular hypotrophy in the absence of hormonal dysfunctions. Semen analysis of one of the subjects revealed extremely severe spermatozoid head abnormalities with necrospermia and severe reduction in velocity [120].

Using Sanger sequencing and targeted re-sequencing, an Italian research group found a novel homozygous mutation in exon 3 of GBA2 in three siblings, a pair of dizygotic twins and a third brother with spastic paraplegia. No enzymology was performed but the change was predicted to be deleterious. Affected members of the family show variable phenotype, characterized by spasticity, mild cerebellar signs and moderate eyelid ptosis with a different degree of severity in the proband (severe spasticity, difficulty in walking and frequent falls) with respect to the two brothers (evidence of clinical signs only at neurological examination). None of these patients had evidence of peripheral neuropathy or showed signs of cataracts or hypogonadism [121].

Marinesco-Sjögren syndrome is an autosomal recessive disorder characterized by cerebellar atrophy with ataxia, early-onset cataracts, hypotonia and muscle weakness. Single nucleotide polymorphism (SNP) chip analysis followed by Exome sequencing identified a 2 bp homozygous deletion in GBA2 in two Norwegian families, with Marinesco-Sjögren syndrome. Enzymatic determination of the GBA2 activity in leucocytes was performed and showed reduced activity of GBA2 corresponding to a residual activity of 7% compared with the mean value of 15 controls. All three patients had significantly increased concentrations of plasma GlcCer similar to that observed in untreated Gaucher patients while in erythrocytes, the concentration was higher [116].

GBA2 has been reported to be down-regulated in melanoma. Inducing expression of GBA2 promoted GlcCer degradation and ceramide generation, followed by an unfolded protein response (UPR) causing apoptosis, subsequent decreased anchorage-independent cell growth, and a 40% reduction *in vivo* tumour growth. This not only demonstrates the anti-tumour activity of GBA2 but provides evidence for the role of non-lysosomal GlcCer breakdown as a source of bioactive ceramide and a mechanistic link between glycolipid catabolism and the UPR/death response of melanoma cells [124]. This may be of significance for GD patients with N370S mutations who have an increased risk of developing solid cancers including melanoma [125]. GBA2 expression has also been shown to be reduced by more than 50% in paediatric brain tumours such as glioblastoma compared to normal brain [126].

Miglustat has shown promise in cystic fibrosis treatment because it reduces the inflammatory response to infection by *P. aeruginosa* and restores F508del-CFTR chloride channel activity. Data demonstrates that the anti-inflammatory effects of Miglustat are likely exerted through inhibition of GBA2 rather than ceramide glucosyl-transferase (GlcCerT) or GBA1. Total β-glucosidase, GBA1 and GBA2 activities were elevated in CF bronchial cells infected by *P. Aeruginosa.* No increased susceptibility to bacterial infections has been identified in patients affected by Gaucher disease, treated with Miglustat or in a mouse model of Sandhoff disease treated with Genz-529648 [127].

1.7 GBA3 (EC 3.2.1.21)

GBA3, or cytosolic beta-glucosidase (EC 3.2.1.21), is a predominantly liver enzyme that efficiently hydrolyzes beta-D-glucoside and beta-D-galactoside, but not any known physiologic beta-glycoside, suggesting that it may be involved in detoxification of plant glycosides [128]. GBA3 was found to have significant neutral glucosylceramidase activity (EC 3.2.1.62), suggesting that it may be involved in a non-lysosomal catabolic pathway of glucosylceramide metabolism [129]. However, others did not find significant glucosylceramidase activity when using natural substrates [130]. No GBA3 activity was found in fibroblasts [131]. There was also no

evidence of GlcCer accumulation when GBA3 was inhibited or any association between GBA3 haplotypes and the severity of GD1 manifestations [130].

1.8 Mechanism of action of GBA1, 2 & 3

GBA1 and GBA2 are both able through transglucosylation to catalyze *in vitro* the transfer of glucosyl-moieties from GlcCer to cholesterol, and vice versa [132]. GBA1, 2 and 3 are retaining β-glucosidases which employ a catalytic mechanism that is commonly referred to as the Koshland double displacement mechanism which involves two amino acid residues (Glu and Asp) residing in the active site. In retaining β-glucosidases, these acidic residues are positioned about 5-6 A apart such that one can act as the nucleophile and the other as a general acid/base catalyst [133]. In the first step of the reaction, called glycosylation, the nucleophile residue (Glu-528 in GBA2) attacks the glucose anomeric centre to create a glucosyl-enzyme intermediate, while the acid/base residue (Asp-678 in GBA2) protonates the glycosidic oxygen, leading to the release of aglycone ($-OCH_3$). In the second step (known as the deglycosylation step), the glycosyl enzyme is hydrolyzed by water, with the other residue now acting as a base catalyst deprotonating the water molecule as it attacks [134].

1.9 Parkinson's disease

1.9.1 Introduction/history

Parkinson's disease (PD) is one the parkinsonian syndromes, a family of related movement disorders characterised by akinesia. The principal parkinsonisms can be divided into alpha-synucleinopathies and tauopathies. Alpha-synucleinopathies include PD, dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) [135]. α-Synuclein is a protein of 140 residues that is predominantly and ubiquitously expressed in the brain and is important for the normal function and integrity of synapses [136].

Parkinsonian tauopathies include corticobasal degeneration (CBD), frontotemporal dementia with Parkinsonism linked to chromosome 17 (FTDP-17) and progressive supranuclear palsy (PSP) [135]. Tau is a microtubule-associated protein, predominantly found in neurons whose major function is to bind to and stabilize microtubules. Tauopathies are neurodegenerative diseases characterised by the observation of hyperphosphorylated, insoluble aggregates of tau in neurons and glia of affected brain regions. The best known example is Alzheimer's disease [137].

Parkinson's disease is a common neurodegenerative disorder that affects over 1% of the population aged over 65 years of age. It is named after James Parkinson who first described it in 1817 when he published "An essay on the shaking palsy" [138]. Parkinson described six patients, three of them noticed casually in the street, and one only observed from a distance, with common symptoms including "involuntary tremulous motion" and "a propensity to bend the trunk forwards, and to pass from a walking to a running pace" [139]. In the 1860s the renowned French physician Dr Jean Martin Charcot at the Salpêtrière hospital in Paris, further described the disease and the symptoms of postural instability, bradykinesia, rigidity and tremor, identified by Dr Parkinson and added hypomimia and micrographia. He recognised that not all patients had tremor and suggested the term Parkinson's disease be used rather than *paralysis agitans* (shaking palsy)[140]. The prevalence of PD rises with age from 1% of those over 60 years of age to 4% of the population over 80. The

mean age of onset is approximately 60 years of age, however 10% of cases are classified as young onset occurring between 20 and 50 years of age [141].

The prevalence of PD is higher in men than women with ratios of 1.1:1. to almost 3:1 being reported [141].

1.9.2 Clinical

Clinical symptoms include hypo- and bradykinesia, rigidity and tremor. Other symptoms include postural instability, dysphagia, speech problems, depression and dementia, autonomic dysfunction and cognitive decline [141].

1.9.3 Diagnosis

The diagnosis of PD is generally based on clinical findings although various forms of brain imaging may be useful. The United Kingdom Parkinson's Disease Society Brain Bank has advocated a set of criteria that should be applied when diagnosing the condition. The Queen Square Brain Bank (QSBB) criteria for the diagnosis of PD requires the finding of bradykinesia and at least one of the following symptoms; muscular rigidity, 4- to 6-Hz resting tremor or postural instability. Supportive criteria include unilateral onset, progressive onset and 70-100% response to levodopa. Exclusion criteria include repeated head injury, repeated strokes and sustained remission [135].

A number of scales have been developed to monitor PD including: The movement Disorder Society's Unified Parkinson's Disease Rating Scale (UPDRS); Non-Motors Symptoms Questionnaire (NMSQuest) and the Hoehn and Yahr scale. The Hoehn and Yahr scale ranges from stage 1 where there is unilateral involvement only with minimal or no functional impairment, to stage 5 where the patient is wheelchair bound or bedridden unless aided [142]. The UDPRS is a more extensive scale assessing over 40 aspects of the disease in four parts covering non-motor experiences of daily living (mentation), motor experiences of daily living, motor function (clinical examination) and motor complications [138].

1.9.4 Treatment

So far no drug has proved to be neuroprotective in PD [135]. The main treatment is the dopamine precursor, levodopa on its own or with a peripheral dopa decarboxylase inhibitor or dopamine agonist. Other treatments include monoamine oxidase type B (MAO-B) inhibitors, glutamate antagonists, anticholinergics and surgery [138]. When a patient is first diagnosed they may not yet feel a need for symptomatic treatment and clinicians may wait until symptoms are affecting daily life before initiating treatment [135].

1.9.5 Causes

Parkinson's disease is a synucleinopathy, a group of various neurodegenerative disorders that share a common pathological lesion comprised of aggregates of α-synuclein protein in vulnerable populations of neurons and glia. Other synucleinopathies include dementia with Lewy bodies (DLB), multiple system atrophy (MSA), and neurodegeneration with brain iron accumulation (NBIA) [143]. Parkinsonian symptoms result from the degeneration of approximately 60% of dopaminergic neurons in the substantia nigra pars compacta (SNpc) leading to a loss of 80% of dopamine in the striatum [144]. Under normal circumstances, dopamine is involved in control of the basal ganglia, which function to facilitate behaviour and movements that are required and appropriate, and to inhibit unwanted or inappropriate movements. The loss of dopamine causes dysfunction of the basal ganglia leading to abnormal motor control, alterations in muscle tone and abnormal involuntary movements, or dyskinesias [145].

Although it had been observed that about 15% of PD patients had a strong family history of PD, it was for a long time thought of as being largely caused by environmental agents. This was supported by the outbreak of post-encephalitic parkinsonism in the early 1900s, the biological effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) identified in 1983 and the finding of a lack of difference in concordance for PD in monozygotic twins compared to dizygotic [146]. This changed with the identification of missense mutations in α-synuclein, encoded by

SNCA identified in large American-Italian family called the Contursi kindred. Subsequently, a triplication of the *SNCA* locus was identified in another family called the Iowa kindred [147]. Genetic forms of the disease now account for up to 20% of cases of PD [144]. This includes autosomal dominant forms such as *PARK1* and Leucine-Rich Repeat Kinase 2 (*LRRK2*), autosomal recessive causes such as *PRKN* and *PINK1* and risk loci such as *GBA* [147, 148]. To date, Genome-wide association studies (GWAS) have led to the discovery of as many as 28 genetic risk loci for sporadic PD. The associated risk variants can be common in the population but convey only a slightly increased risk of developing PD. If all known risk factors are considered together, they are only associated with an odds ratio of 3 to 4 and therefore explain only a part of the expected heritability of PD [149].

Various molecular mechanisms have been suggested or identified for the loss of dopaminergic neurons in PD including mitochondrial impairment, oxidative stress, ubiquitin–proteasome system (UPS) dysfunction and altered calcium homeostasis [144].

Evidence for mitochondrial involvement include the findings of a significant loss of mitochondrial complex I in SNpc of PD brains [150], and the PD symptoms caused by complex I inhibition by MPTP [151, 152]. See chapter five for further discussion.

1.10 Gaucher Parkinson's

1.10.1 Introduction

Since the report of six GD1 patients with Parkinson's disease in 1996 it has been recognised that there is an association between PD and GD [153]. In 2004, it was reported that Parkinsonism was more common in GD carriers than the general population [154]. A later prospective study estimated that the risk of PD in GD1 patients was 21 times greater than the general population [155]. A large meta-analysis of 5691 PD patients and 4898 controls from 16 centres around the world found that the odds ratio for any *GBA* mutation in PD patients versus controls is 5.43. PD Patients with GBA1 mutations were more likely have affected relatives and presented earlier with the disease than those without [156]. GD heterozygote

status is now also known to be the strongest genetic risk factor for PD [157]. GBA1 heterozygotes with non N370S mutations associated with GD have an increased risk of PD compared to those with N370S mutations [157]. The p.E326K polymorphism in GBA1 which does not cause GD is as significant risk factor as classic GD mutations for developing PD [158]. While GBA1 associated PD is most common in the Ashkenazi Jewish population, GBA1 mutations are a significant risk factor for developing PD across the world including Belgium [159], Canada [160], China [161], Korea [162], Sweden [163] and Thailand [164]. The differing risk found in different populations and centres may reflect differences in ethnicity but also the methods used and mutations screened for [156]. The exact risk for GD patients developing PD is not known, but has been estimated as 20- to 30-fold while 5–10% of PD patients have GBA mutations, making them numerically the most important risk factor for the disease identified to date [165]. In the UK, GBA1 mutations are found at a higher frequency than any other known Parkinson's disease gene [166].

A Genome-Wide Association Studies (GWAS) of 418 patients with clinical diagnoses of PD and 306 unaffected and unrelated control subjects found the largest risk effect for variants in GBA, with odds ratios (OR) of 4.46 (p= 0.05) for rs76763715 (N370S, MAF 0.00665) and 4.98 (p=0.009) for rs75548401 (K26R, MAF 0.01173)[167]. Another GWAS study of 478 PD patients and 337 healthy individuals also found mutations in GBA to be the most common risk factor with 11% of the PD patients and 4.5 % controls carrying heterozygous variants in the GBA gene leading to an OR of 2.28 (p = 0.0007). This compares to an OR of 1.86 (p=0.01) for *LRRK2* variants [168].

While the majority of GD patients with PD present with Gaucher symptoms, some patients have been diagnosed with GD after a diagnosis of PD [169]. As a result, it is now becoming routine to screen patients with Parkinsonism for GD. However, while the incidence of Parkinsonism is higher in GD1 patients than reference populations, the majority of Gaucher patients do not develop PD. The likelihood of an individual patient with GD1 developing Parkinsonism before the age of 70 years is only 5% to 7% compared to the incidence of Parkinsonism in the general population estimated

at 0.3% in the entire population, and 1% in those over 60 years of age [87]. The vast majority of GD carriers also never develop PD [170].

1.10.2 Clinical presentation

It is not possible to discriminate individual PD patients with GBA1 mutations (PD-GBA1) from those with idiopathic PD (iPD) [171]. Patients with PD-GBA1 present with the classic PD symptoms of asymmetric tremor, rigidity and bradykinesia [172, 173]. At the point of diagnosis, patients with PD-GBA1 mutations appear clinically indistinguishable from idiopathic disease, however the risk of progression to dementia is more than five times that of iPD patients [174, 175]. PD-GBA1 patients also showed a 4-fold increase in the risk of progression to Hoehn and Yahr stage 3, an end-point associated with impaired quality of life [175]. The age at onset (AAO) of GD patients with PD has found to be earlier with a mean of 49.7 years compared to GD heterozygotes with PD with a mean AAO of 54.9 years and iPD patients with a mean AAO of 62.4 years [176]. Pain, particularly shoulder pain, has been found to be a significantly more frequent presenting symptom in PD-GBA1 (10.3%) than in iPD (3%) [173].

As well as the documented anosmia in PD patients including PD-GBA1, It is known that the ability to discriminate colour is poor in PD patients. Interestingly, it has been found that GD patients have better colour discrimination than healthy controls and although colour discrimination is reduced in GD patients with PD and GD carriers with PD, both groups have significantly better colour discrimination than those with iPD [177].

1.10.3 Pathophysiology

Various theories that have been proposed to explain the link between GD and PD. It has been suggested that:-

- Misfolded GBA1 directly contributes to α-synuclein accumulation.

Mutant GBA1 mutations, resulting in misfolded mutant protein may contribute to the enhanced aggregation of α-synuclein directly, by a biochemical interaction with

α-synuclein [178]. This is supported by the finding of significant co-localization between GBA1 and α-synuclein in Lewy neurites and α-synuclein positive inclusions in GD homozygotes with PD with 90% of Lewy bodies showing a positive signal for GBA1, compared to 75% in GD heterozygotes and only 10% of PD patients without GBA1 mutations [179]. It has also been demonstrated using cellular and *in vivo* models that mutant GBA1 promotes α-synuclein accumulation in a dose- and time-dependent manner [180]. Another theory is that the accumulation of GlcCer in neurons due to GBA1 deficiency promotes the formation of toxic α-synuclein oligomers triggering further depletion of GBA1 and further stabilization of the α-synuclein oligomers by GlcCer accumulation, resulting in a self-propagating positive feedback loop leading to neurodegeneration [181].

- Misfolded GBA1 burdens the autophagy or lysosomal systems.

α-synuclein is degraded in part by chaperone-mediated autophagy (CMA) and also by the lysosome. Disruption to autophagy or lysosomal systems either directly by mutant GBA1 or by accumulating substrate due to GBA1 deficiency could impair α-synuclein elimination. Alternatively the elimination of damaged mitochondria by mitophagy could be compromised although there is no experimental evidence of this in PD-GBA1 [178].

- GBA1 mutations impair endoplasmic reticulum retention associated degradation (ERAD).

It has been shown that GBA1 variants present variable degrees of ERAD. The authors proposed that unlike wild type protein, mutant GBA1 is a substrate for parkin-mediated ERAD degradation. Mutant GBA1 interaction with parkin could block interactions with other parkin substrates, interfering with their UPS mediated breakdown, which could lead to ER stress and eventual cell death [182].

- GlcCer accumulation alters lipid metabolism in GD.

Under normal conditions, α-synuclein co-localizes with lipid rafts that mediate its delivery to the synapse, but under conditions of altered lipid metabolism, this

association is disrupted. This leads to a redistribution of the protein to the cell body from neurites, which could lead to the formation of abnormal and potentially toxic α-synuclein species. A disruption of normal α-synuclein–lipid interactions due to diminished GBA1 activity could represent a pathway that leads to cellular death [183].

- Macrophage proliferation enables the prionic spread of α-synuclein.

It has been demonstrated that α-synuclein aggregation is transmitted between neurons in the brain raising the possibility that PD is a prion disease. And is has been suggested that Gaucher cells could accumulate α-synuclein, which could acquire the prion form, which may be accidently transported or released by the cell [184].

None of the mechanisms that involve mutant/misfolded GBA protein explain why PD has been described in GD patients with null mutations i.e. with no protein product at all. Theories involving the accumulation of substrate (GlcCer or glucosylsphingosine) do not explain why GD carriers who should not be accumulating unhydrolysed substrate have a higher risk of developing PD.

The clinical heterogeneity seen in GD has been observed with regards to PD in a pair of monozygotic brothers, who were heterozygous for the N370S mutation, where one was diagnosed with PD at age 63, while no signs or symptoms were found in his twin after detailed clinical examination [185].

1.10.4 Treatment

Most studies have shown that patients with PD-GBA1 have a good response to l-DOPA, similar to those with iPD [169]. Enzyme replacement therapy in GD patients does not cross the blood-brain barrier and does not seem to have any effect on the progression of parkinsonian symptoms [169]. However, substrate reduction therapy may prove useful. The effects of PD-linked GBA mutations were reversed by the molecular chaperones ambroxol and isofagomine in human cell and fly models, providing proof of principle that small molecule chaperones can reverse

mutant GBA-mediated ER stress *in vivo* and might prove effective for treating PD [186].

1.11 GBA1 association with other movement disorders

There is a significant association between GBA1 mutation carrier status and dementia with Lewy bodies (DLB) with an odds ratio of 8.28 (confidence interval 4.78-14.88). The same group found an odds ratio for GBA1 and PD with dementia (PDD) of 6.48 (confidence interval 2.53-15.37) [187]. GBA mutations have been found at autopsy in 23% of brain samples of cases with DLB [188] and it has also been reported that there is a decrease in GBA activity in cerebrospinal fluid of DLB patients [189].

Multiple system atrophy (MSA) is a progressive neurodegenerative disorder, characterized by autonomic failure, poor levodopa-responsive parkinsonism, cerebellar ataxia, and various pyramidal symptoms [143]. There is conflicting evidence with regards the association of GBA1 mutations and multiple system atrophy (MSA). Three groups found no association [169], while another found that mutations in GBA1 are a risk factor for MSA but with an odds ratio of 2.43 (confidence interval 1.14-5.21) the risk factor is lower than that for PD [190].

Neurodegeneration with brain iron accumulation (NBIA) comprises a spectrum of progressive extrapyramidal disorders characterized by high levels of iron accumulation in the brain. There is no correlation with GBA1 and NBIA reported in the literature [143].

1.12 Alzheimer's disease and GBA1

Significant reductions of GBA1 expression and enzyme activity has been reported in the brain of patients with Alzheimer disease (AD) suggesting that this deficiency could play a role in the development of AD by inducing lysosomal dysfunction although the mechanism is unknown. Over-expression of GBA1 promoted the lysosomal degradation of Abeta1-42 oligomers, restored the lysosomal impairment, and protected against the toxicity in neurons treated with Abeta1-42 oligomers

[191]. However, GBA1 measurement in amygdala from AD patients found no difference in activity compared to controls [192].

1.13 Case study - Atypical parkinsonism with apraxia and supranuclear gaze abnormalities in type 1 Gaucher disease.

This is a summary of a previously reported case of a genetically proven GD1 patient with an atypical parkinsonian corticobasal degeneration (CBD) like syndrome [193].

The patient, a male of Ashkenazi Jewish ancestry was diagnosed with GD1 at the age of 17 after the finding of asymptomatic mild thrombocytopenia and hepatosplenomegaly. He did not receive treatment for his GD. He had no further symptoms until the age 60, when he experienced difficulty in performing complex intellectual tasks and forgetfulness. He then developed problems with reading and writing and became apathetic, fearful, socially withdrawn and developed depression. On examination, "there was facial hypomimia and global bradykinesia. Speech was hypophonic, agrammatic and non-fluent. He had apraxia of eyelid opening and closure, bilateral gestural dyspraxia, grasping and magnetism, and abnormal posturing of his right arm, with bilateral stimulus sensitive myoclonus to touch. There was difficulty in initiating eye movements and a supranuclear gaze abnormality with restriction and hypometric saccades, especially in the vertical plane. Right predominant cog-wheel rigidity and bradykinesia of the limbs was noted. Gait was slow and short stepped, with occasional freezing and impaired postural reflexes." Apart from mild neutropenia and thrombocytopenia, routine blood tests were normal. Genetic testing was negative for *LRRK2* and tau gene mutations [193]. See paper in appendix for further details.

Testing performed in our laboratory summarised in **Table 1.2** revealed that leucocyte GBA1 activity was very low and consistent with a diagnosis of GD. Plasma chitotriosidase was elevated which is consistent with the diagnosis of GD but the level was much lower than usually observed in untreated GD. The probably reflects the very mild GD phenotype exhibited by the patient. GBA gene analysis showed he was a compound heterozygote for the N370S/L444P (p.Asn409Ser/p.Leu483Pro) GBA1 mutations. Analysis of CSF monoamine metabolites revealed marked

impairment of not just dopamine but also serotonin turnover demonstrated by undetectable levels of the dopamine metabolite homovanillic acid (HVA) and the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA).

Table 1.2 - Laboratory results of a GD patient with atypical parkinsonism.

Leucocyte GBA1	**0.4** (5.4-16.8 nmol/hr/mg protein)
Plasma chitotriosidase	**623** (0-150 nmol/L/hr)
Mutation	**N370S/L444P**
CSF HVA	**<0.5** (71 – 565 nmol/L)
CSF 5-HIAA	**<0.5** (58 – 220 nmol/L)

Regarding the low 5-HIAA, there are reports of low serotonin and its metabolites in parkinsonism but not as profound as observed in this patient. It has been suggested that l-Dopa treatment stimulates serotonergic neurons to preferentially release dopamine thereby reducing serotonin release, so reduced 5-HIAA levels in CSF might be expected to reflect l-Dopa treatment [194]. Herbert *et al*., observed that l-Dopa treated PD patients had lower levels of 5-HIAA than non l-Dopa treated PD patients. However, the levels of 5-HIAA were also reduced in the l-Dopa treated PD patients compared to controls. Reduced 5-HIAA levels in PD patients could therefore reflect intrinsic serotonergic deficits in PD which may be exacerbated by l-Dopa treatment [195]. Another group reported significantly reduced CSF levels of both serotonin and 5-HIAA in PD patients compared to age-matched control subjects, and patients with Alzheimer's disease. However, no correlation emerged between serotonin/5-HIAA concentrations and UPDRS-III, disease duration or age and levels did not correlate with the presence of depression, apathy or sleep disturbance [196].

There is only one report in the literature investigating GBA1 mutations and CBD. An Italian study of 2766 unrelated consecutive patients with a clinical diagnosis of primary degenerative parkinsonism including 34 patients with CBD, and 1111 controls confirmed the association of GBA1 mutations with PD and DLB, but found no association with CBD or PSP. The authors suggest that GBA1 dysfunction is relevant for synucleinopathies, such as PD and DLB, but not for tauopathies such as CBD and PSP [197]. However, while the finding of CBD in a GD1 patient could be coincidental, this case study raises the possibility of an association between GD and atypical parkinsonism such as CBD.

1.14 Other Lysosomal storage disorders and PD

While the link between GD and PD is now well established there is evidence that other LSDs may also have a connection to PD. Features of PD have been described in five of the six most common LSDs, including metachromatic leucodystrophy, mucopolysaccharidosis IIIA, Fabry disease and mucopolysaccharidosis II as well as GD [198]. A decrease in the enzyme activity and protein levels of alpha-galactosidase, the enzyme associated with Fabry disease has been found in sporadic PD [199]. A decrease in the enzyme activity of arylsulphatase A, the enzyme associated with metachromatic leucodystrophy has been found in familial parkinsonism [200]. Parkinsonian tremor has been described in a heterozygote for Niemann-Pick C disease [201]. Other connections between common neurological diseases and rare LSDs are being identified. Mutations in the gene that codes for progranulin (*GRN)* one of the genes which is long-known to cause frontotemporal dementia when mutated in one of its alleles, were recently shown to cause a novel LSD, neuronal Ceroid lipofuscinosis 11 (CLN11), when affecting both its copies [202].

A candidate-gene study of 347 Greek patients with sporadic PD and 329 healthy controls conducted to investigate the association between 5 polymorphisms in the *SCARB2* gene and the development of PD revealed an association for the rs6825004 polymorphism [203]. This association was also reported in a DLB study [204]. The largest case-control genome-wide association study (GWAS) of PD based on a single

collection of individuals to date (3,426 cases and 29,624 controls discovered a novel genome-wide significant association with PD-rs6812193 near *SCARB2* (p = 7.6 x 10(-10), OR = 0.84) [205]. Others were not able to replicate the association with rs6825004 but confirmed the association with rs6812193. They also found that the increased risk is not mediated by changes in GBA1 activity [206]. However, a study of the relative RNA expression by real-time PCR, and LIMP-2 levels on Western blots failed to demonstrate any appreciable difference in *SCARB2* expression and LIMP-2 levels among samples after grouping by rs6812193 or rs6825004 genotypes suggesting that the two reported SNPs may not be related to *SCARB2* and demonstrate the challenges in interpreting some association studies [207]. While further work is required to confirm the risk of PD associated with *SCARB2*, evidence form LIMP2 deficient mice has demonstrated that LIMP-2 expression is critical for GBA1 activity and α-synuclein clearance [208].

Loss-of-function mutations in *ATP13A2*, the gene encoding P-type ATPase, responsible for lysosomal acidification causes Kufor-Rakeb syndrome (*PARK9*), an autosomal recessive form of early-onset parkinsonism with pyramidal degeneration and dementia [209]. And mutations in the vacuolar protein sorting 35 homolog (VPS35) gene at the *PARK17* locus, which encodes a protein involved in endosomal-lysosomal trafficking has been identified as causative of a late-onset PD [210, 211]. However, no association was found between the D620N mutation of the VPS35 gene and PD in a study of 124 patients in Hungary [212].

1.15 Hypotheses

Mutations in GBA1 are the hereditary basis of GD and are a risk factor for developing PD. The vast phenotypic variations among patients with GD including those with the same genotype and the discordant GD phenotypes observed in some monozygotic twins demonstrate the complexity of the disorder and supports a role for genetic modifiers [23, 90-93, 95-97]. This equally applies to the situation where, although they have a significantly higher risk of doing so than the general population, most GD patients and carriers do not develop Parkinson's disease [87,

170]. GBA2 is a non-lysosomal beta-glucosidase capable of hydrolysing the same substrates as GBA1 [134] and could be a modifying factor in GD and PD-GBA1.

1.16 Aims

1. To document the prevalence of GBA1 insufficiency in patients being investigated for Parkinsonism or dystonia.
2. To develop and optimise methods for the measurement of GBA1 and GBA2 activities in various tissue types from clinical samples and model systems.
3. To use these methods to document GBA1 and GBA2 activities in various tissue types from clinical samples and model systems.
4. To document the downstream consequences of inhibition of GBA1 and/or GBA2.
5. To determine if GBA1 is susceptible to oxidative stress and the effects of oxidative stress on GBA1.

Chapter 2 Materials and general methods

2.1 Reagents

The following materials were purchased from Sigma-Aldrich (Poole, UK).

Bicinchoninic acid; BSA protein standard; 4 % (w/v) Copper (II) sulphate solution; N-butyldeoxynojrimicin; conduritol β epoxide; 3-methyldopa; acetyl-Coenzyme A; Oxaloacetate; and 4-methylumbelliferyl-β-d-galactopyranoside; In Vitro Toxicology Assay Kit, MTT based; Dimethyl sulfoxide (DMSO); Glycine; Sodium hydroxide; Trizma® base (Tris); 4–methylumbelliferone; Ethylenediaminetetraacetic acid tetrasodium salt dehydrate (EDTA); 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-gluco-pyranoside;4–methyl-umbelliferone; PBS (pH 7.4) P3813-10PAK.

The following materials were purchased from VWR international (Lutterworth, UK)

2-Methoxyethanol; Acetic acid; Citric acid; Sodium phosphate; Citrate phosphate; sodium acetate.

The following materials were purchased from ThermoFisher Life Technologies Ltd (Paisley, UK)

Dulbecco's Modified Eagle's Medium/Ham's F-12 nutrient mixture (DMEM/F12); L-glutamine; Hanks' Balanced Salt Solution without calcium; magnesium, or phenol red (HBSS); 0.25% Trypsin-EDTA; Trypan Blue stain (0.4%); C-Chip disposable haemocytometer; MycoFluor™ Mycoplasma Detection Kit; 4X LDS NP0007, 10X DTT NP0004, NuPAGE antioxidant NP0005, NuPAGE antioxidant NP0005, 20X MOPS (3-(N-morpholino)propanesulfonic acid) running buffer (dilute to 1X for use) NP0001, Magic Mark LC5602, SeeBlue Plus 2 (Rainbow Marker) LC5925, SimplyBlue SafeStain LC6060, NuPAGE 4-12% Bis-Tris Gel NP0322BOX, Iblot Transfer Stack PVDF Mini IB401002.

The following materials were purchased from Labtech International Limited (Uckfield, UK)

Fetal bovine serum (FBS).

The following materials were purchased from Melford (Ipswich, UK)

4-methyl-β-d-glucopyranoside; 4-methyl-α-d-glucopyranoside.

The following materials were purchased from Merck Millipore (Watford, UK)

Taurocholic Acid, Sodium Salt, ULTROL® Grade.

The following materials were purchased from ThermoFisher Scientific (Paisley, UK)

Triton™ X-100 Surfact-Amps™ Detergent Solution.

The following materials were purchased from Toronto Research Chemicals

Acarbose.

The following materials were purchased from DAKO

10X TBST (Tris-buffered saline tween-20) S3306.

The following materials were purchased from Abcam

Anti-GBA (reacts with human GBA1, 500 µg/mL) ab55080, Anti-GBA2 (reacts with human GBA2, 500 µg/mL) ab205064.

The following materials were purchased from New England Biolabs

Blue Pre-stained Protein Standard, Broad Range (11-190 kDa) ladder P7706S.

The following materials were purchased from Santa Cruz

Donkey anti-rabbit IgG-HRP (400 µg/mL) sc-2313, Goat anti-mouse IgG-HRP (400 µg/mL) sc-2005, Anti- GAPDH (FL-335) (reacts with human GAPDH, 200 µg/mL): sc-25778.

The following materials were purchased from Thermo Scientific

IP Lysis Buffer 87787, Halt Protease Inhibitor (PI) Cocktail 78410, EDTA 78410, Inhibitor Solution 78410, SuperSignal West Pico Chemiluminescent Substrate 34080, ECL (Enhanced chemiluminescence) Reagents 1 & 2 (part of a kit) 32209.

2.2 Samples

All human patient samples and controls were processed and stored in accordance with Royal College of Pathologists guidelines.

2.3 Plasma and leucocyte preparation.

Blood was collected into lithium heparin tubes which were centrifuged at 1625 RCF at +4^{o}C for ten minutes. The plasma was removed into a labelled 2.5 mL tube and fast frozen in dry ice/methanol and then stored at -20^{o}C until required.

Leucocytes were obtained from blood using water to preferentially lyse red cells from the pellet. The buffy coat (white cell layer) was removed from the red cells after the removal of the plasma, into a labelled 2.5 mL tube to which some 0.9% saline had been added. This was stored on ice while the original blood tube was centrifuged again at 1625 RCF at +4^{o}C for ten minutes after the addition of 0.9% saline and mixing. Any cells remaining at the buffy coat layer after the second centrifugation were removed and added to the tube with first cells harvested. This tube was filled with 0.9% saline and centrifuged at 1625 RCF at +4^{o}C for five minutes. Then all the liquid was removed and 1.5 mL of cold distilled water was added and the tube agitated for 90 seconds, after which 0.5 mL of 3.6% saline was added to return the osmolality to isotonicity. This tube was centrifuged at 1625 RCF for 10 seconds to preferentially precipitate the intact leucocytes. The liquid above

was removed and the process was repeated a second time to clean up the leucocyte pellet. If necessary a third lysis step was carried out. Once a satisfactory leucocyte pellet was obtained, it was washed once in 0.9% saline, the saline removed after centrifugation and then 50 μL of water was added and the pellet was fast frozen in dry ice/methanol and then stored at -20^{o}C until required.

2.4 Prepared Reagents

2.4.1 Buffers

MV Buffers

A solution of 0.1 M citric acid was prepared by dissolving 21.0 g of citric acid in 1L of deionised H_20 (dd H_20). A solution of 0.2 M di-sodium hydrogen phosphate (Na_2HPO_4) was prepared by dissolving 28.4 g of di-sodium hydrogen phosphate in 1L of deionised H_20. These stock solutions were stored at -20^{o}C until required.

MV 4.0 buffer was prepared by adding 90 mL of 0.1 M citric acid to 60 mL of 0.2 M Na_2HPO_4 and adjusting where necessary until the pH was exactly 4.0.

MV 4.1 buffer was prepared by adding 90 mL of 0.1 M citric acid to 60 mL of 0.2M Na_2HPO_4 and adjusting where necessary until the pH was exactly 4.1.

MV 4.5 buffer was prepared by adding 108 mL of 0.1 M citric acid to 92 mL of 0.2M Na_2HPO_4 and adjusting where necessary until the pH was exactly 4.5.

MV 5.4 buffer was prepared by adding 88 mL of 0.1 M citric acid to 112 mL of 0.2M Na_2HPO_4 and adjusting where necessary until the pH was exactly 5.4.

For the pH curves a range of buffers of different pH were prepared by mixing the appropriate amount of 0.1 M citric acid to 0.2M Na_2HPO_4 and adjusting where necessary until the desired pH was obtained.

Acetate buffers

Acetate buffer pH 3.8 was prepared by adding 58 μL of glacial acetic acid to 8mL of deionised H_20, adjusting pH to 3.8 with 1mol/L NaOH and then making up to 10 mL with deionised H_20.

Acetate buffer pH 4.95 was prepared by adding 29 μL of glacial acetic acid to 8mL of deionised H_20, adjusting pH to 4.95 with 1mol/L NaOH and then making up to 10 mL with deionised H_20.

Acetate buffer pH 6.5 was prepared by adding 58 μL of glacial acetic acid to 8mL of deionised H_20, adjusting pH to 6.5 with 1mol/L NaOH and then making up to 10 mL with deionised H_20.

The in use working buffers were stored at +4^{o}C and stocks were stored at -20^{o}C until required.

2.4.2 0.25M Glycine-NaOH pH10.4 stopping reagent

A 1M glycine/NaOH solution was prepared by adding 64 g NaOH to 200 mL of deionised H_20, and keeping cooled on ice while stirring until completely dissolved and the solution was cool. 150 g of glycine was dissolved in 1600 mL of deionised H_20. The NaOH solution was slowly added to the glycine solution, with stirring until the pH reached 10.4. The volume was made up to 2 litres with deionised H_20. Working 0.25M glycine/NaOH stopping reagent was prepared by taking 250 mL of 1M glycine/NaOH and making the volume up to one litre with deionised H_20. Working 0.25M glycine/NaOH was stored at room temperature.

2.4.3 4-methylumbelliferone standard

A 200 μM stock solution of 4-methylumbelliferone (4-MU) was prepared by dissolving 17.6 mg of 4-MU in a few mL of ethanol and then making up to 500mL with deionised H_20. This was then filtered through a 0.22 μM filter and aliquoted into sterile universals. Working solution was prepared by taking 5 mL of 200 μM stock and making up to 20 mL with deionised H_20 to give a 50 μm solution. Then 5

mL of 50 μM solution was made up to 50 mL with deionised H_20 to give a 5 μM working standard. 200 μL of the working standard was then aliquoted into 1.5 mL Eppendorf tubes, equivalent to 1 nm of 4-MU per tube. Both stock and working standards were stored at -20^{o}C until required. When required an aliquot of working standard was thawed and 1 mL of Glycine/NaOH stopping reagent added.

2.5 Lysosomal enzyme assays

After rapid thawing at +37 ^{0} C, samples were disrupted using a Soniprep 150 ultrasonic disintegrator (MSE UK Ltd.) for 10 seconds at amplitude 8A for leucocytes or 6A for cultured cells.

Protein was determined using the BCA method (see 2.11).

2.6 Beta-glucosidase (GBA1, EC 3.2.1.45)

At acid pH β–glucosidase hydrolyses the substrate 4–methylumbelliferyl–β–D–glucopyranoside to 4–methylumbelliferone and glucose. Adding sodium taurocholate activates lysosomal beta-glucosidase and inhibits the cytosolic beta-glucosidase which is very active. Adding alkaline buffer stops the enzyme reaction and causes 4–methylumbelliferone to fluoresce at a different wavelength from unhydrolysed substrate, thereby permitting its measurement in the presence of a vast excess of unhydrolysed substrate.

2.6.1 Leucocyte GBA1

Samples were assayed in duplicate. Each tube contained 80 μL of 37.2 mmol/L sodium taurocholate (NaT) and up to 20 μL of sample containing 60 μg of protein. The volume was made up to 100 μL using deionised water. Substrate blanks were prepared with 80 μL of the NaT and 20 μL deionised water. At timed intervals 100 μL of substrate (5 mmol/L in McIlvaine citrate-phosphate buffer pH 5.4 (MV5.4) was added to each tube, which was briefly vortexed before incubating at +37 ^{o} C for 60 minutes. Following the incubation, the reaction was stopped by the addition of 1.0 mL 0.25 M glycine/NaOH buffer pH 10.4 before reading on the LS55 fluorimeter (Perkin Elmer) at excitation 365 nm, emission 450 nm [213]. The amount of free 4–

methylumbelliferone (4-MU) released, corresponding to the nanomoles of substrate hydrolysed was calculated using a standard containing 1nmol of free 4-MU in 200 μL H_2O to which 1.0 mL 0.25 M glycine/NaOH buffer (pH 10.4) had been added.

2.6.2 Cultured cells GBA1

A portion of the sonicated sample was diluted in deionised water to give a protein concentration of 1 mg/mL. Samples were assayed in duplicate. Each tube contained 25 μL of MV 5.4, 15 μL of 148.8 mmol/L sodium taurocholate (NaT) and 10 μL of sample. Substrate blanks were prepared as above but with 10 μL of deionised water instead of sample. At timed intervals 50 μL of substrate (10mM in deionised water) was added to each tube, which was briefly vortexed before incubating at +37 ^{o} C for 60 minutes. Following the incubation, the reaction was stopped by the addition of 1.1 mL 0.25 M glycine/NaOH buffer pH 10.4 before reading on the LS55 fluorimeter (Perkin Elmer).

2.7 Beta-galactosidase (EC 3.2.1.23)

The lysosomal enzyme, b-galactosidase was assayed using the synthetic fluorescent substrate 4-methylumbelliferyl-β-d-galactopyranoside, which is cleaved by the enzyme into 4-methylumbelliferone and galactose.

2.7.1 Leucocyte beta-galactosidase

Each tube contained 40 μL of McIlvaine citrate-phosphate buffer pH 4.1 (MV 4.1), 5 μL of 0.2 M potassium chloride and 5 μL of sample. Substrate blanks were prepared with 40 μL of the buffer, 5 μL of 0.2 M potassium chloride and 5 μL of deionised water. At timed intervals 150 μL of substrate (1mM in MV 4.1) was added to each tube, which was briefly vortexed before incubating at +37^{o} C for 15 minutes. Following the incubation, the reaction was stopped by the addition of 1 mL of 0.25 M glycine/NaOH buffer pH 10.4 before reading on the LS55 fluorimeter (Perkin Elmer) [214].

2.7.2 Cultured cells beta-galactosidase

Cultured cells were assayed as above but with each sample tube containing 7 mg of protein and the volume made up to 50 μL with 0.4% human serum albumin (HSA) in 0.4 M sodium chloride.

2.8 Total β-hexosaminidase (EC 3.2.1.51)

The lysosomal enzyme total beta-hexosaminidase was assayed using the synthetic fluorescent substrate 4-methylumbelliferyl-N-acetyl-β-D-glucosamine, which is cleaved by the enzyme into N-acetyl-β-D-glucosamine and the fluorescent 4-methylumbelliferone.

2.8.1 Cultured cells

A portion of the sonicated sample was diluted in deionised water to give a protein concentration of 1 mg / mL. For each sample 5 μL of the 1mg/mL diluted enzyme was added to 500 μL 0.2% Human Serum Albumin (HSA) in McIlvaine citrate–phosphate pH 4.5 (MV 4.5) buffer (HSA/MV 4.5), mixed and kept on ice. Samples were assayed in duplicate. Each tube contained 100 μL of the sample in HSA/MV 4.5. Substrate blanks were prepared with 100 μL of HSA/MV 4.5. All tubes were pre-incubated for two minutes. Then at timed intervals 100 μL of substrate was added to each tube, which was briefly vortexed before incubating at +37 ^{o} C for 10 minutes. Following the incubation, the reaction was stopped by the addition of 1.0 mL 0.25 M glycine/NaOH buffer pH 10.4 before the fluorescence was read at excitation 365 nm, emission 450 nm on the LS55 fluorimeter (Perkin Elmer).

2.8.2 Leucocytes

A portion of sonicated sample was added to an equal volume of 0.4M potassium chloride and the sample frozen in dry ice/methanol and the thawed at +37 ^{o} C three times. The sample was then centrifuged at 1625 RCF for 5 minutes at +4 ^{o} C. For each sample 5 μL of the centrifuged supernatant, was added to 500 μL MV 4.5 buffer, mixed and kept on ice and assayed as above.

2.8.3 Plasma total beta-hexosaminidase

For each sample 5 μL of plasma was added to 95 μL McIlvaine citrate–phosphate pH 4.5 (MV 4.5) and then assayed in duplicate as above except sample were incubated for twenty minutes.

2.9 Acid α-1,4-glucosidase (EC 3.2.1.20) in cultured cells

At acid pH β–glucosidase hydrolyses the substrate 4–methylumbelliferyl–α–D–glucopyranoside to 4–methylumbelliferone and glucose. Adding alkaline buffer stops the enzyme reaction and causes 4–methylumbelliferone to fluoresce at a different wavelength from unhydrolysed substrate, thereby permitting its measurement in the presence of a vast excess of unhydrolysed substrate.

Samples were assayed in duplicate. Each tube contained 90 μL of Acetate buffer pH 3.8 and 10 μL of sample. Substrate blanks were prepared with 90 μL of the buffer and 10 μL of deionised water. At timed intervals 10 μL of substrate (60mM in 2-Methoxyethanol) was added to each tube, which was briefly vortexed before incubating at +37 ^{o} C for 30 minutes. Following the incubation, the reaction was stopped by the addition of 1.08 mL of 0.25 M glycine/NaOH buffer pH 10.4 before reading on the LS55 fluorimeter (Perkin Elmer).

2.10 Citrate synthase (EC 2.3.3.1)

Citrate synthase catalyses the condensation of oxaloacetate and acetyl-coenzyme A to form citric acid and coenzyme A in the first step of the Krebs's cycle. This takes place in the mitochondrial matrix. Citrate synthase is commonly used as a measure of mitochondrial activity [215, 216]. The citrate synthase assay is based on the method of Shepherd and Garland 1969 [217]. The assay measures the production of coenzyme A as a result of a reaction between free coenzyme A with 5,5'-Dithio-bis (nitrobenzoic acid (DNTB).

Sample was added (20μL) to a 1.5 mL cuvette containing 950 μL of 100mM Tris buffer pH 8 with 0.1 % V/V Triton, 10 μL of 10 mM Acetyl-Co-A and 10 μL of 20 mM DTNB. A reference for each sample was set up with set up in the same way but with 960 μL of Tris buffer. The reagents were mixed gently by inversion. An initial baseline reading was obtained by measuring absorbance for two minutes before the reaction was started by the addition of 10 μL of 20 mmol/L oxaloacetate to the sample cuvettes. The cuvettes were incubated at +30^{o}C and the reaction was measured at 412 nm for 10 minutes at thirty second intervals on a Uvikon 922 spectrophotometer (Biotek instruments). The absorbance difference between the sample and reference cuvette for each sample was converted to molar concentration using the Beer-Lambert law. The extinction coefficient used for DTNB was 13.6 x 10^{3} M^{-1} cm^{-1} (path length 1cm, volume 1mL). Results are expressed as nmol/min/mg protein. In order to validate the assay, the linearity between citrate synthase activity and the protein concentration was determined. A control homogenate of known protein concentration was diluted to give a range between of 0.5 mg/mL and 2.5 mg/mL. The assay was linear up to 2.5 mg/mL but for assay all samples were diluted to 1mg/mL (**Figure 2.1**).

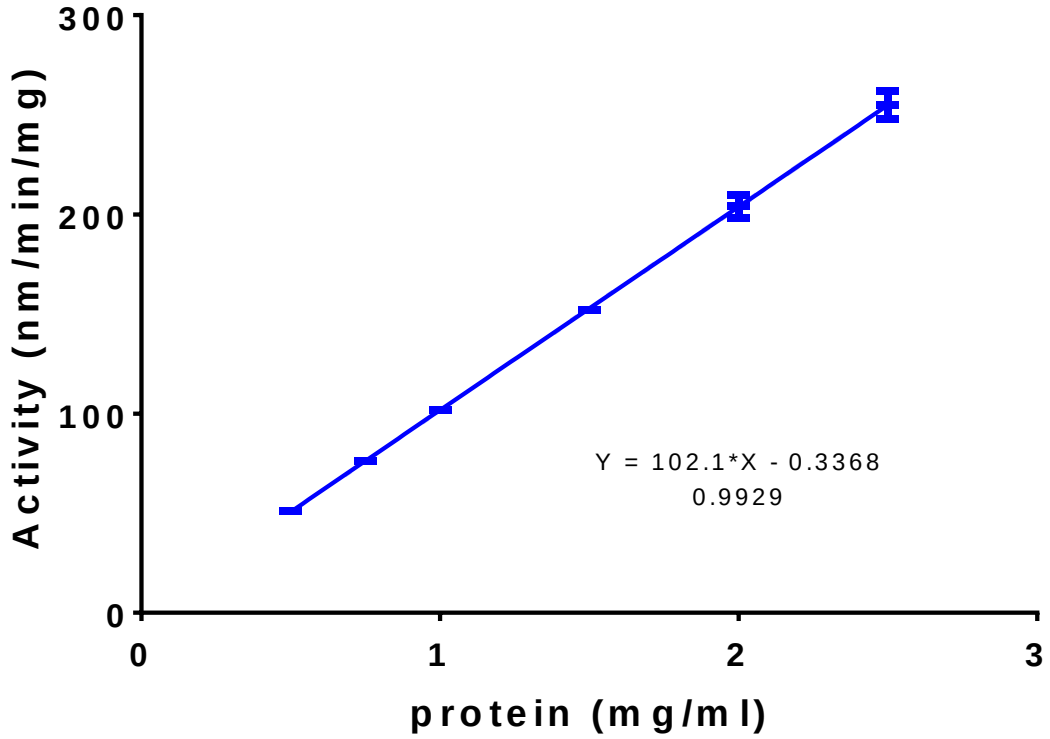

Figure 2.1 - A plot to show the relationship between citrate synthase activity and protein concentration.

The assay was linear up to 2.5 mg/mL, R2 = 0.993, n=3.

2.11 Total Protein assay

Total protein was determined by the bicinchoninic acid assay (BCA) assay [218] using bovine serum albumin as a standard. Bicinchoninic acid (BCA) sodium salt, is a stable, water-soluble compound which forms a purple complex with cuprous ion (Cu1+) in an alkaline environment. The reaction combines the well-known biuret reaction, where protein reacts with Cu^{2+} in alkaline medium to produce Cu^{1+}. Two molecules of BCA react with one of Cu^{1+} to form a purple colour with maximal absorbance at 562 nm.

To 50 μL of sample or standard, 1 mL of bicinchoninic acid was added and incubated at +37 ^{o} C for 10 minutes after vortexing. Then 20 μL of 4 % (w/v) Copper (II) sulphate solution was added and incubated at +37 ^{o} C for 20 minutes after vortexing. Absorbance was read on a Cecil CE2041 spectrophotometer at 562 nm. Sample protein concentration was calculated from the BSA standard curve (0-50mg/mL). While the assay was shown to be linear up to 200mg/mL (**Figure 2.2**), the routine standard curve used was 0-50mg/mL. If the absorbance of a sample was lower than the lowest standard (5mg/mL), the assay was repeated with a larger sample volume and likewise if the absorbance reading was higher than the top standard (50 mg/mL), the assay was repeated with a smaller volume. The majority of the assays used a sample volume between 2.5 μL and 20 μL with the volume made up to 50 μL with deionised water.

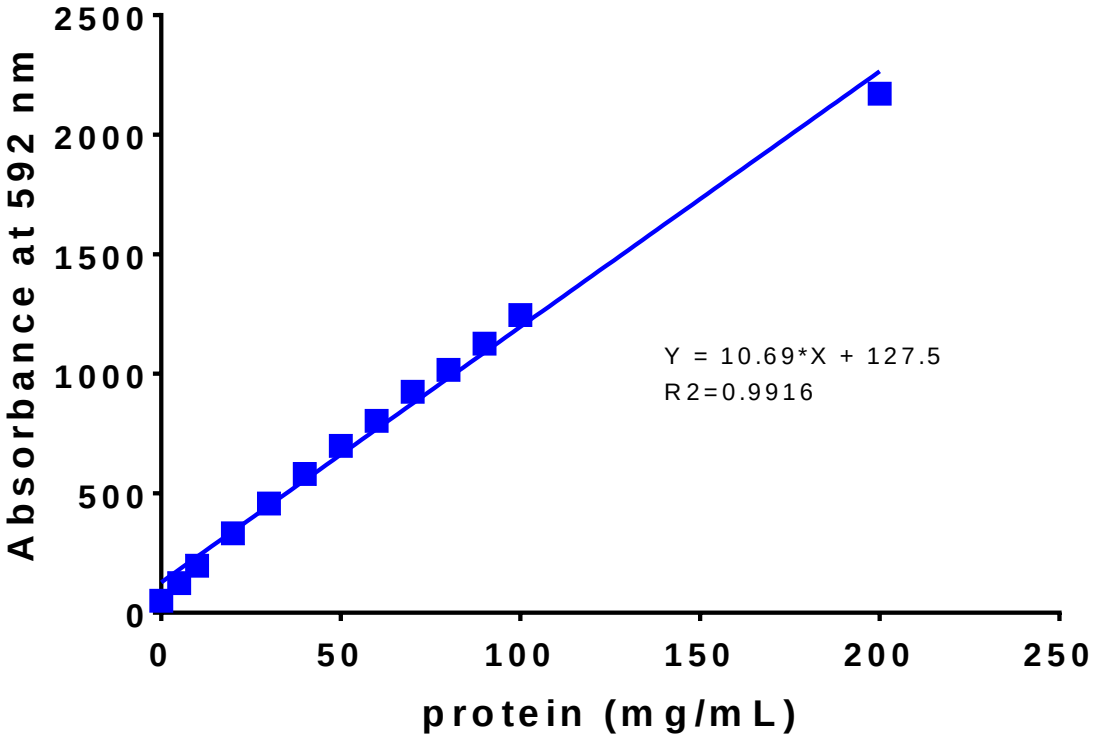

Figure 2.2 - BCA protein standard curve.

The protein assay is linear up to 200mg/mL, R2 = 0.992, n=3.

2.12 Cell culture

2.12.1 SH-SY5Y cells

The SH-SY5Y cell line has become a popular cell model for PD research because this cell line possesses many characteristics of dopaminergic neurons [219]. These cells express tyrosine hydroxylase and dopamine-β-hydroxylase, as well as the dopamine transporter [220]. Moreover, this cell line can be differentiated into a functionally mature neuronal phenotype in the presence of various agents. SH-SY5Y was thrice-cloned originally from SK-N-SH and first reported in 1978. A neuroblast-like sub-clone of SK-N-SH, named SH-SY, was sub-cloned as SH-SY5, which was sub-cloned again as SH-SY5Y. This cell line is genetically female (has two X chromosomes, but no Y), as the original line was established in 1970 from a bone marrow biopsy of a metastatic neuroblastoma site in a four year-old female [221].

2.12.1.1 Cell seeding and passage

SH-SH5Y cells were obtained from the European Collection of Cell Cultures (Health Protection Agency, Salisbury, UK) and cultured in accordance with the supplier's instructions.

Cells were seeded at 1 x 10^4 cells/cm^2 in a 75 cm^2 tissue culture flask in working medium i.e. Dulbecco's Modified Eagle's Medium/Ham's F-12 nutrient mixture (DMEM/F-12) supplemented with 100mL/L fetal bovine serum (FBS) and 5 mmol/L L-glutamine. Cells were grown in 10mL of working medium at +37^{O}C in 5% CO_2. Cell culture medium was replaced the day after seeding and every 48 hours thereafter. Cells were passaged at 80-90 % confluence approximately every 6 days.

To passage the cells, the medium was removed from the flask and the cells were washed with 6mL of Hanks' Balanced Salt Solution (HBSS). After removing the HBSS, cells were detached with 4mL/flask of 0.25% trypsin-EDTA incubated at +37^{O}C for 3 minutes. The 0.25% trypsin-EDTA was diluted down with the addition of 8mL of medium.

The detached cells could then be prepared for storage, passaged into one or more flasks or harvested for assays.

2.12.1.2 Cell storage

The cells were passaged as described above, harvested and centrifuged at 500 g for five minutes at +4 ^{O}C. The supernatant was removed and the cells suspended in working medium. An aliquot of cell suspension was mixed 1:1 with 0.4% Trypan blue and cells were counted manually using a C-Chip disposable haemocytometer. Cells were stored in 1mL aliquots in cryovials at a density of 1 x 10^6 cells/mL in freezing medium containing 700 mL/L 1:1 DMEM/F-12 with 200 mL/L FBS and 100 mL/L Dimethyl sulfoxide (DMSO). The aliquots were frozen overnight in a -80^{O}C freezer before being transferred into liquid nitrogen.

The cells arrived at passage 16 and after culture 18 vials of 1mL of cells in freezing medium were stored at passage 19. A fresh aliquot was reconstituted for each experiment.

2.12.1.3 Cell recovery

Cells were recovered from liquid nitrogen, thawed rapidly in a +37 ^{O}C waterbath and quickly seeded at a density of 1 x 10^4 cells/cm^2 in 75 cm^2 flasks and then cultured and passaged as previously described.

2.12.1.4 Cell harvesting and washing

The cells were passaged as described above, harvested and centrifuged at 500 g for five minutes at +4 ^{O}C. The supernatant was removed and the cells were re-suspended in 4mL 0.9% sodium chloride. The cells were centrifuged again at 500 g for five minutes at +4 ^{O}C. The supernatant removed and the cells were re-suspended in another 4mL 0.9% sodium chloride. This was repeated again. After the third wash in 4mL 0.9% sodium chloride, the supernatant was removed from the centrifuged cells and 50 μL of deionised water added. The cells were then quickly frozen in a dry ice/methanol cooling bath before being store at -20 ^{O}C until assay. All enzyme assays were performed at passage 22-24.

2.12.1.5 Mycoplasma testing

Mycoplasma infections are relatively common in laboratory cell cultures and are difficult to detect as they are not visible to the naked eye or under routine light microscopy. Mycoplasma infections, which are typically difficult to detect during routine cell culture work, can cause physiological and morphological distortions that affect experimental results [222]. The MycoFluor™ Mycoplasma Detection Kit couples the use of a fluorescent nucleic acid stain with fluorescence microscopy [223].

Cultured cells were tested for mycoplasma as per the kit instructions, when they were been passaged for storage or just before a final experiment. When being passaged, 2-3 drops of cell suspension was placed on a sterile cover slip in a sterile Petri dish and 4 mL of working tissue culture medium. The cells were grown until approximately but no more than 75% confluence. Fixative solution (3:1 (v/v) solution of 100% methanol:glacial acetic acid) was prepared fresh on the day of use. Fixative equivalent to the volume of medium in the Petri dish i.e. 4mL was gently added directly to the medium containing coverslip. After about 5 minutes, the solution of fixative and medium was removed and 4 mL of fresh fixative added. After an additional 10 minutes, the fixative was removed and the coverslip gently washed twice with deionised water. Working MycoFluor™ reagent was prepared by adding 1 volume of 20X concentrated MycoFluor™ reagent to 19 volumes of deionised water. The coverslip was covered in the Working MycoFluor™ reagent for 10 minutes shielded from light. The coverslip was then removed with forceps, held vertically, and the excess medium that accumulated on its edge gently blotted. The coverslip was then placed cell side down, onto a clean microscope slide and sealed using the coverslip sealant provided in the kit. Once dry, the slide was examined on a Nikon Eclipse Ci fluorescent microscope, excitation 365 nm and band-pass 450 nm using a 100X oil immersion objective.

2.12.2 Fibroblasts culture

Fibroblasts were cultured and processed as per the SH-SY5Y cells except the culture medium was HAMS F10, with 12% fetal calf serum and 1% penicillin/streptomycin.

2.13 Statistical analysis

The statistical significance of data sets was assessed using Student's t-test where there were two sets of data. Comparisons were made using a one way analysis of variance (ANOVA) followed by a Newman-Keuls multiple comparison test, where there were more than two sets. $P<0.05$ was considered significant.

All graphs display the mean ± the standard error of the mean (SEM), unless otherwise stated.

Chapter 3 Characterisation of GBA1 and GBA2 activities in various tissues.

3.1 INTRODUCTION

There is a growing interest in the potential role of GBA1 and GBA2 in modifying the disease phenotype of GD and the pathogenesis of PD [224-226]. In order to evaluate the potential interplay between these two enzymes and their potential contributions to GD/PD, reliable enzyme assays are required.

As discussed previously in chapter 1, although GBA1 and GBA 2 are both glucosylceramidases, the two enzymes act on different glycolipids, do not share sequence identity and are expressed in different tissues and subcellular compartments. They respond differently to inhibitors, summarized in **Table 3.1**. This has been used in the routine measurement of GBA1 in leucocytes which is measured in the presence of sodium taurocholate which activates GBA1 and inhibits GBA2 [213]. These different responses to inhibitors can be exploited to enable the measurement of both GBA1 and GBA2 using the artificial fluorescent 4-Methylumbelliferyl β-D-glucopyranoside substrate.

Table 3.1 - A comparison of the properties of GBA1 and GBA2.

	GBA1	GBA2
Chromosome	1	9
Location	Lysosome	Non-lysosomal
Inhibited by CBE	Yes	No
Inhibited by NBDNJ	No	Yes
Inhibited by NAT	No	Yes
Activated by NAT	Yes	No

AIMS

Whilst methods for the determination of GBA1 in a range of cell types and tissues have been developed often using the synthetic substrate, 4–methylumbelliferyl–β–D–glucopyranoside, there is less data available regarding GBA2 activity. Consequently, prior to assessment of these enzymes in clinical samples and model systems, protocols were developed here for the reliable measurement of GBA1 and GBA2 using brain as the most relevant tissue and leucocytes as an easy accessible sample type.

Two separate approaches to measuring GBA2 were investigated. The first using the GBA1 inhibitor CBE, with the activity remaining being non-lysosomal beta-glucocerebrosidase i.e. GBA2. And the second approach, using the GBA2 inhibitor, with GBA2 activity calculated as the difference in activity with and without NBDNJ inhibition.

Following the establishment of the assays, the relative activities of the two enzymes were recorded in a range of tissue and cell types to ascertain whether there is evidence of the differing metabolic roles of the enzymes across the samples studied.

In this study, brain, leucocytes, plasma, cultured fibroblasts, and astrocyte (1321N) and neuronal cell (SH-SY5Y) lines were studied.

3.2 Methods and materials

3.2.1 Brain

Brains from mixed sex CD1 mice were removed and stored at −80 °C until assayed for this study. The brains were supplied by Matthew Gegg (Department of Clinical Neuroscience, UCL Institute of Neurology, Hampstead Campus, Rowland Hill Street, London). Mouse procedures and welfare were approved by the University College London Animal Welfare and Ethical Review Board (AWERB) and in accordance with project and personal licenses granted by the UK Home Office and the Animal (Scientific Procedures) Act of 1986.

Brain samples were homogenised in water as a 2.5% brain homogenate using a mini glass homogeniser. Protein levels were determined using the BCA method and the sample diluted in water to a protein concentration of 2 mg/mL.

3.2.2 Leucocytes and plasma

Leucocytes and plasma were isolated from lithium heparin blood samples and prepared by the method described previously in section 2.3. The samples had been collected after clinical consent for clinical investigation from patients at Great Ormond Street Hospital for Children NHS foundation Trust. After rapid thawing at +37 ^{0} C, leucocyte pellets were sonicated with a Soniprep 150 ultrasonic disintegrator (MSE UK Ltd.) for 10 seconds at amplitude 8A. A pooled leucocyte preparation was prepared by combining sample remaining after routine assay from twenty leucocyte pellets from patients who had been found to have unaffected levels of the lysosomal enzymes that had been measured in those samples.

3.2.3 SH-SY5Y cells and 1321N1cells

SH-SY5Y cells from were cultured and harvested as described previously in section 2.12.1. The 1321N1 cell line is a human astrocytoma cell line isolated in 1972 as a sub clone of the cell line 1181N1 which in turn was isolated from the parent line U-118 MG, one of a number of cell lines derived from malignant gliomas [227]. Harvested 1321N1 cells were kindly provided by Matthew Gegg from the Royal Free Hospital.

3.2.4 Cultured fibroblasts

Fibroblasts from disease controls were cultured and harvested as described previously in section 2.12.2. The samples had been collected after clinical consent for clinical investigation from patients at Great Ormond Street Hospital for Children NHS foundation Trust. Samples were processed and stored in accordance with Royal College of Pathologists guidelines, UK. Control samples were from patients who had lysosomal enzymes measured as part of their investigations but were not found to have a deficiency of a lysosomal enzyme.

3.2.5 Cerebrospinal fluid

Discarded anonymised Cerebrospinal fluid (CSF) was kindly provided by the Neurometabolic unit at The National Hospital for Neurology and Neurosurgery. The CSF had been through one freezer thaw cycle prior to storage at -80^{o}C.

3.2.6 Control lysosomal enzyme activities

The activities of beta-galactosidase, total beta-hexosaminidase and alpha-glucosidase were determined as control enzymes as described in chapter 2**.** The assay of control enzymes is to determine if any changes observed in GBA1 were due to an overall change in lysosomal content or activity and to exclude any samples that had become degraded from analysis.

3.2.7 Protein determination

Sample protein concentration was determined using the BCA protein method described in section 2.11.

3.3 Experimental protocol and results of method development in brain

3.3.1 Feasibility experiment to determine if beta-glucosidase activities are measureable in brain

As an initial experiment a 2.5% homogenate of human brain was prepared and beta-glucosidase was assayed using the fibroblast GBA1 method described previously in section 2.6.2 but substituting water or various inhibitors for NaT. The sample was assayed with no inhibitor, with 1mM CBE, with 148.8 mmol/L NaT and with both 1mM CBE and 148.8 mmol/L NaT. Beta-galactosidase was measured as per the leucocyte assay described in section 2.7.1 to ascertain its usefulness as a control enzyme in brain.

The initial feasibility experiment showed that beta-glucosidase activity was measurable in brain **(Table 3.2)**. The addition of sodium taurocholate led to a significant 72% decrease in activity ($P<0.01$) whereas the addition of CBE led to a smaller but still significant 28% decrease ($P<0.01$) indicating that GBA1 is not the main source of beta-glucosidase activity in brain. Beta-galactosidase activity was 73.3 nmol/hr/mg ptn and easily measurable using the leucocyte assay and so can be used as a control enzyme when comparing different samples/groups.

Table 3.2 - **Beta-glucosidase activities in a 2.5% homogenate of brain.**

Inhibitor	Activity measured	Beta-glucosidase Activity (nmol/hr/mg ptn)
None	Total inactivated	48.18 ± 0.07
NaT	Activated lysosomal	13.50 ± 0.5
1mM CBE	GBA1 inhibited	34.5 ± 0.5
1mM CBE & 148.8 mmol/L NaT	GBA1 & 2 inhibited	1.57 ± 0.10

3.3.2 pH curves for beta-glucosidase activities in brain

To ascertain the optimum pH to measure non-lysosomal beta-glucosidase, a 2.5 % homogenate of brain in water was assayed in triplicate as described previously with 10 μL of sample but 70 μL of the appropriate pH buffer in each tube rather than water and the substrate was made up in water. The buffers ranged from pH 4.6 to pH 7.0. Blanks were set up in duplicate for each pH as per samples but substituting water for sample. Each pH point was assayed under 4 conditions; with no inhibitor, with 1mM CBE, with 148.8 mmol/L NaT and with both 1mM CBE and 148.8 mmol/L NaT.

The maximum activity under all conditions was observed at pH 5.6 indicating that the *in vitro* pH optimum for both lysosomal and non-lysosomal beta-glucosidase in brain against the artificial substrate is essentially the same under these assay conditions (**Figure 3.1**).

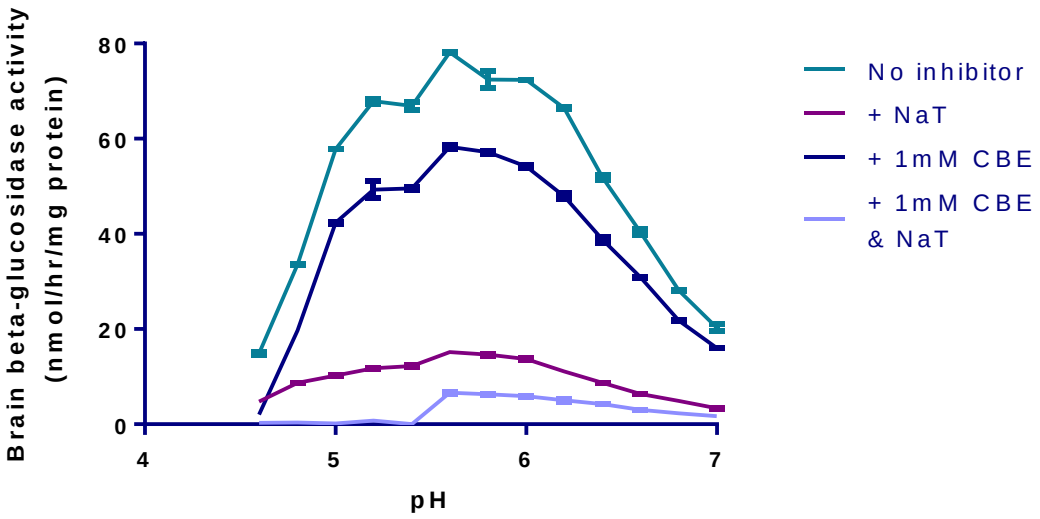

Figure 3.1 -pH curve for brain beta-glucosidase (pH 4.6-7.0).

Sample assayed with no inhibitor, 1mM CBE, 148.8 mmol/L NaT and with both 1mM CBE and 148.8 mmol/L NaT. The maximum activity under all conditions was observed at pH 5.6.

To confirm the initial findings the experiment was repeated as before but with more pH points over the range of interest i.e. pH 5.0-5.8. Additionally the samples were assayed with 2.5 μM of the GBA2 inhibitor NBDNJ.

When the experiment was repeated with more pH points between pH 5.0 to 5.8, the maximum activity without any inhibitor was at pH 5.6. The maximum activity in the presence of the GBA1 inhibitor CBE was at pH 5.2 but there was no significant difference in the activity at any pH from 5.0 to 5.6 (**Figure 3.2**).

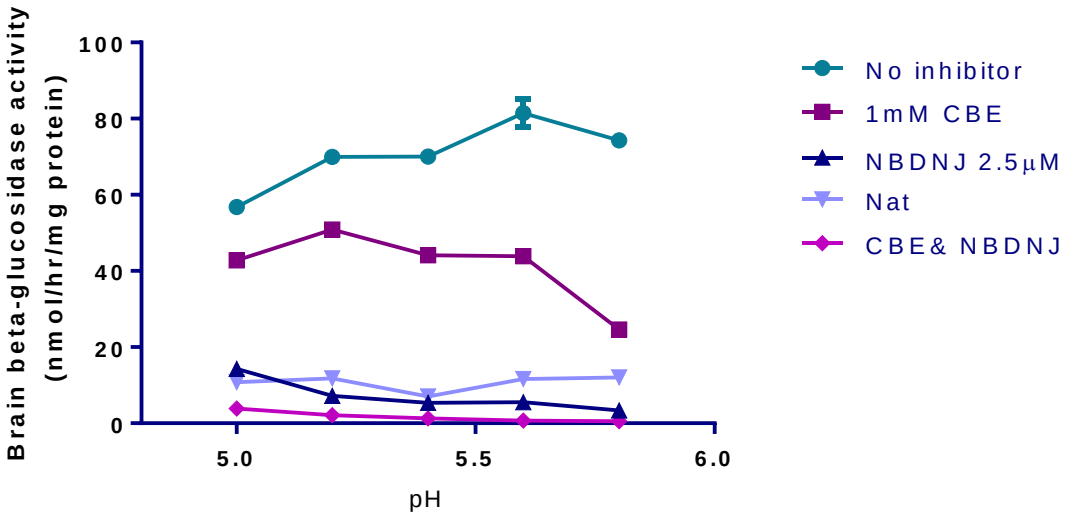

Figure 3.2 - Brain beta-glucosidase activities at various pH (5.0-5.8)

Assayed with no inhibitor, 1mM CBE, 2.5 μM NBDNJ, 148.8 mmol/L NaT and with both 1mM CBE and 2.5 μM NBDNJ.

3.3.3 CBE inhibition of brain beta-glucosidase activity

To ascertain the optimum concentration of CBE to inhibit lysosomal beta-glucosidase and the time required for inhibition to happen, 2.5% brain homogenate was assayed as previously described with a range of CBE concentrations from 0-5mM. CBE or water was added to the samples which were left to incubate on ice for times varying from 2-60 minutes before assay. Blanks were set up for each CBE concentration and incubation time as per the samples but with the addition of water instead of sample.

Increasing concentrations of CBE led to increasing inhibition of beta- glucosidase activity, however inhibition at each concentration did not change significantly with increasing time of inhibition before assay indicating that it is not time dependent (**Figure 3.3**).

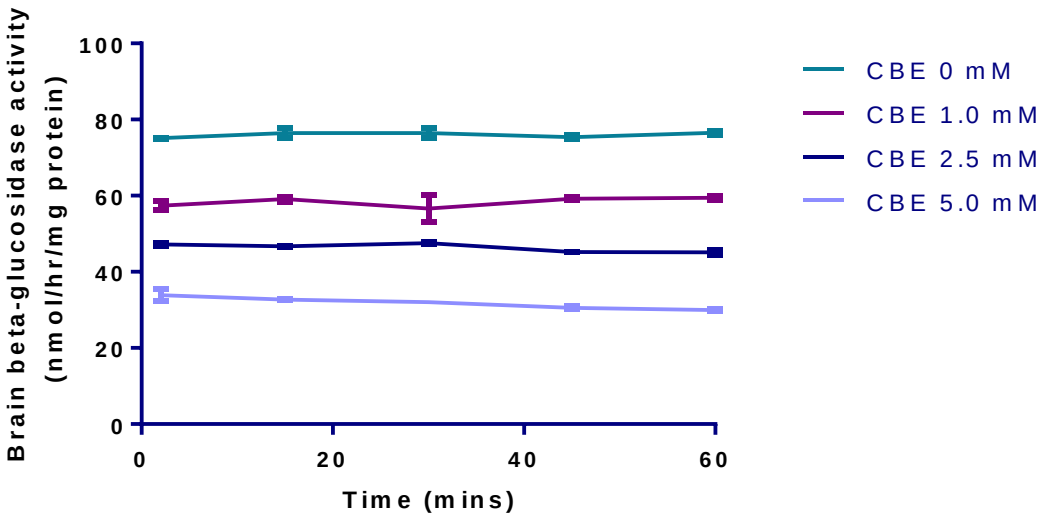

Figure 3.3 - Brain beta-glucosidase activities with varying concentrations of CBE.

3.3.4 NBDNJ inhibition of brain beta-glucosidase in the presence of CBE

Three sets of tubes were set up in triplicate with 10 μL of 2.5% brain homogenate and NBDNJ varying from 0-25 μM in the tube. One set contained of tubes contained no CBE, one set 1.0 mM CBE and the third set 5.0 mM CBE. Blanks were set up for each condition with water replacing sample. Beta-glucosidase activity was measured as previously described.

There was an 88% decrease in beta-glucosidase activity when assayed with 2.5 μM NBDNJ in the absence of CBE. Increasing NBDNJ had a negligible effect (**Figure 3.4**). Assaying with 2.5 μM of the GBA2 inhibitor NBDNJ led to a 87.6% decrease in the measurable beta-glucosidase activity whereas increasing concentrations of the GBA1 inhibitor CBE led to greater inhibition with almost 50% decrease in activity with 5.0 mM CBE. This would indicate that one of the inhibitors is not specific.

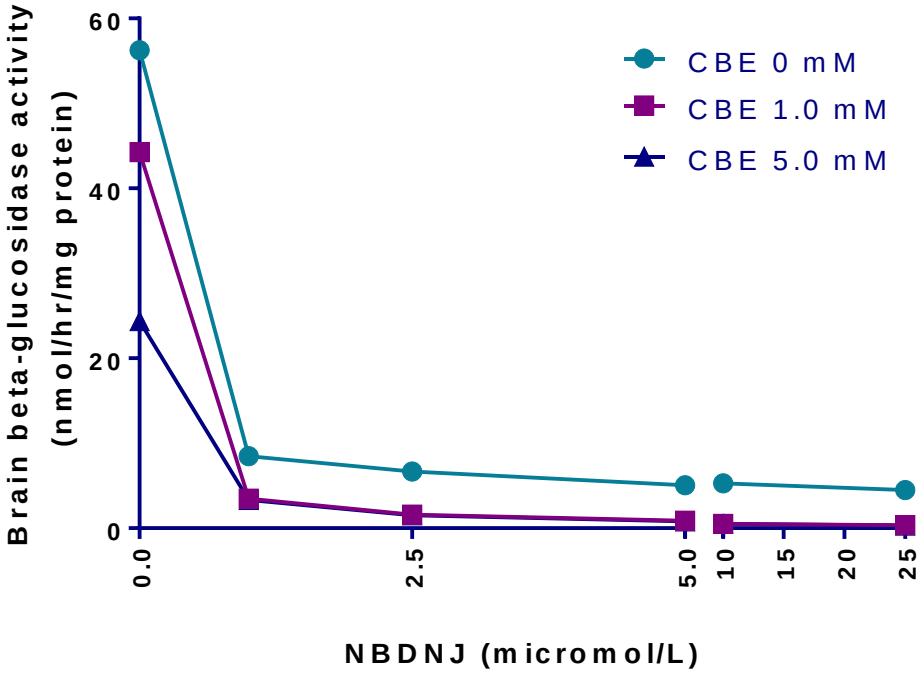

Figure 3.4 - Brain beta-glucosidase activities with varying concentrations of NBDNJ in the presence of none, 1mM or 5.0 mm CBE.

3.3.5 CBE inhibition in brain in the presence of NBDNJ

Two sets of tubes were set up in triplicate with 10 μL of 2.5% brain homogenate and CBE varying from 0-2.0 mM in the tube. One set contained of tubes contained 2.5 mm NBDNJ and the other set the same volume of water. Blanks were set up for each condition with water replacing sample. Beta-glucosidase activity was measured as previously described.

In the absence of NBDNJ there was a continuing decrease in beta-glucosidase activity with increasing concentrations of CBE (**Figure 3.5**). However, beta-glucosidase activity was reduced by 97% in the presence of 0.2 mM CBE when assayed with 2.5 μM NBDNJ compared to the activity with neither inhibitor. There was no further decrease in activity with increasing CBE concentration in the presence of NBDNJ.

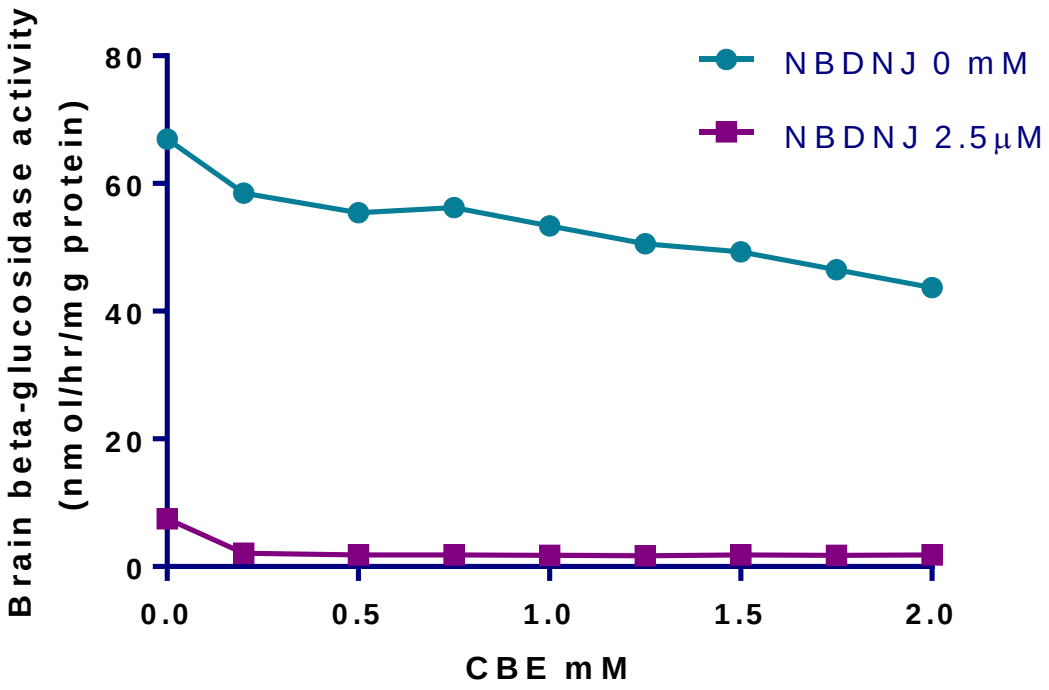

Figure 3.5 - Brain beta-glucosidase activities with varying concentrations of CBE in the presence of none or 2.5 μM NBDNJ.

3.3.6 The effect of protein concentration on brain beta-glucosidase activity

Two sets of tubes were set up in triplicate with varying amounts of 2.5% brain homogenate which had been diluted to either 0.2mg/mL or 2.0 mg/mL protein. This gave a range of protein from 0.0025 mg/tube to 0.25 mg/tube. One set of tubes contained 2.5 μM NBDNJ and the other set the same volume of water. Blanks were set up for each condition with water replacing sample. Beta-glucosidase activity was measured as previously described.

Increasing protein concentrations led to a steady increase in fluorescence and activity/tube over the range examined (0-0.25mg/mL) with and without the presence of NBDNJ (**Figure 3.6**). However, the rate of reaction peaked at 0.05mg/mL with and without the presence of NBDNJ (**Figure 3.7**). Experiments to date have had a protein of 0.1mg/mL which is on the linear part of the curve. Samples with proteins above 0.15 mg/mL had high fluorescence readings requiring dilutions to obtain a valid reading. Future experiments and assays will be done with a protein of 0.1mg/mL in the reaction tube equivalent to 10 μL of a 2mg protein/mL sample in a final assay volume of 200 μl.

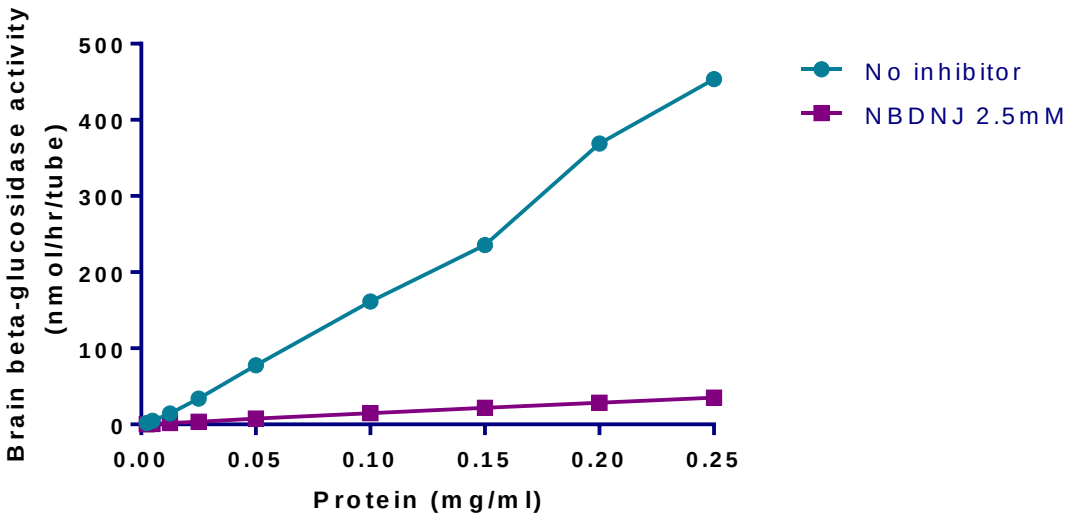

Figure 3.6 - Brain beta-glucosidase activities expressed as nmol/hr/tube with varying protein levels in the presence of none or 2.5 μM NBDNJ.

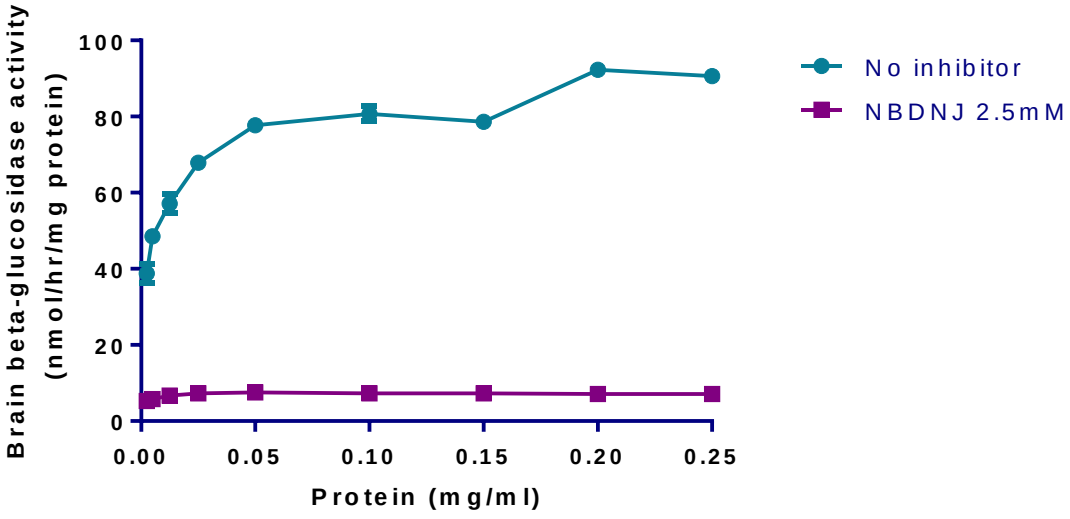

Figure 3.7 - Brain beta-glucosidase activities expressed as nmol/hr/mg protein with varying protein levels in the presence of none or 2.5 μM NBDNJ.

3.3.7 The effect of substrate concentration on brain beta-glucosidase activity

To ascertain the optimum substrate concentration, a 2.5 % homogenate of brain in water was assayed in triplicate as previously with substrate ranging from 1mM to 20mM. Blanks were set up in duplicate for each substrate concentration as per samples but substituting water for sample. Each substrate concentration was assayed with either no inhibitor or 2.5 μM NBDNJ.

Enzyme activity had reached maximum velocity by 10mM substrate (**Figure 3.8**). Experiments to date have used 10mM substrate and future experiments and assays will be done at this concentration.

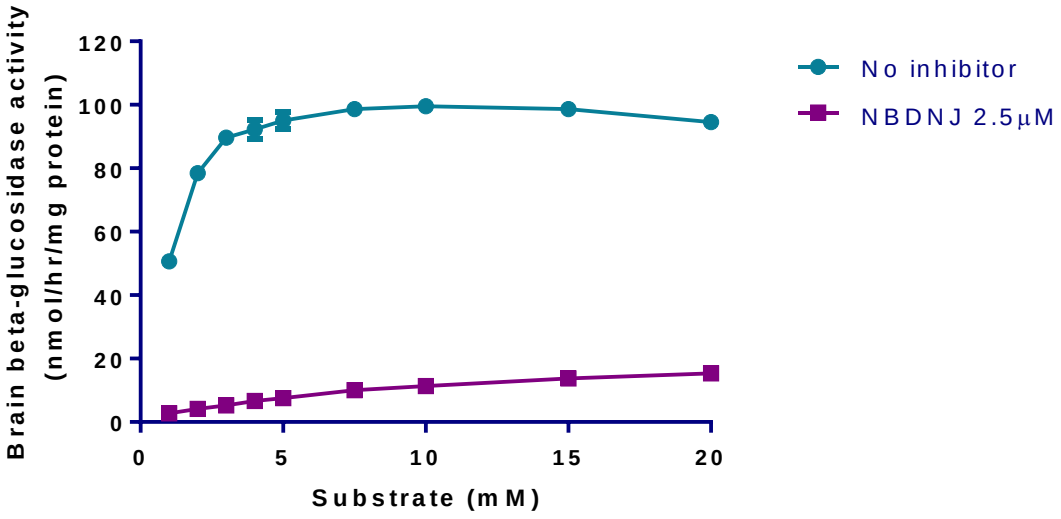

Figure 3.8- Brain beta-glucosidase activities with varying substrate concentrations in the presence of none or 2.5 μM NBDNJ.

The experiment was repeated with 1mM CBE or with 148.8 mmol/L NaT (**Figure 3.9**). Again, enzyme activity had reached maximum velocity by 10mM substrate.

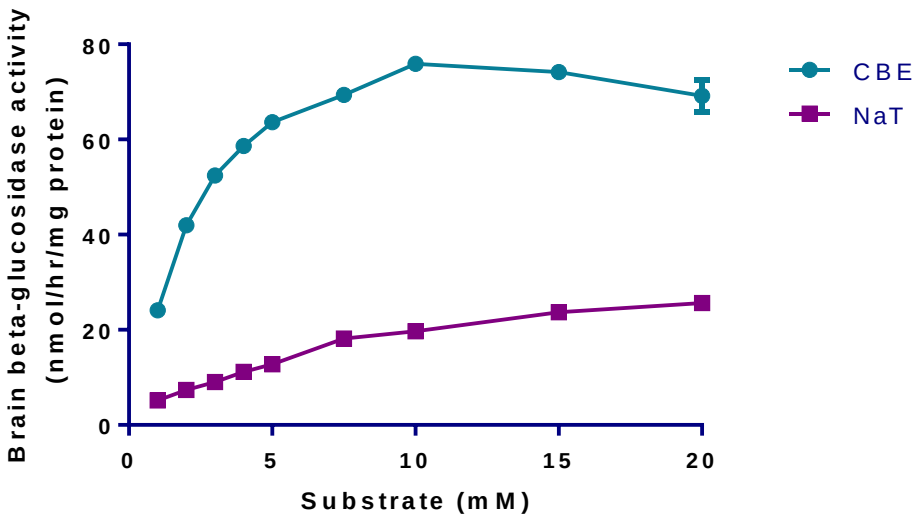

Figure 3.9 - Brain beta-glucosidase activities with varying substrate concentrations in the presence of 1 mM CBE or 149 mM Sodium taurocholate.

3.3.8 The effects of CBE and NBDNJ on fluorescence

To ascertain if the presence of either CBE or NBDNJ had any quenching effects or auto-fluorescence, four sets of standards were prepared in triplicate as described in section 2.4.3 with one set containing no inhibitor, one set 25 μM NBDNJ, one set 5.0 mM CBE and one set 25 μM NBDNJ and 5.0 mM CBE.

There was no evidence that either CBE and/or NBDNJ had any quenching effect or auto-fluorescence at levels higher than used in any of the experiments (**Table 3.3**). This confirms that any change in enzyme activities is due to inhibition of enzyme activity.

Table 3.3 - Fluorescence of 4-MU standard with no inhibitor, 25 μM NBDNJ, 5.0 mM CBE or 25 μM NBDNJ and 5.0 mM CBE.

	No Inhibitor	**25 μM NBDNJ**	**5.0 mM CBE**	**25 μM NBDNJ and 5.0 mM CBE**
Fluorescence units	437 ± 0.6	440 ± 3.1	436 ± 0.6	440.3 ± 1.9

3.3.9 Summary of brain GBA1 and GBA2 assays.

GB1 and GBA2 are both measurable in brain. GBA2 is the predominant beta-glucosidase activity in brain.

GBA1 and GBA2 are measureable in brain by homogenising brain as a 5% homogenate, measuring the protein and diluting down to 2mg/mL protein in water. 10 μL of 2 mg/mL sample is incubated with 100 μL of 10mM 4–methylumbelliferyl–β–D–glucopyranoside substrate in MV buffer pH 5.4 for +37 ^{o} C for 60 minutes before stopping with 1.0 mL 0.25 M glycine/NaOH buffer (pH 10.4) and reading the fluorescence at excitation 365 nm, emission 450 nm. GBA1 is measured by performing the assay with 148.8 mmol/L sodium taurocholate, whereas GBA2 is measured by performing the assay with and without 2.5 μmol NBDNJ.

The same techniques used to develop the assays in brain are next used to investigate GBA2 activity in leucocytes, based on the established leucocyte GBA1 assay described in section 2.6.1.

3.4 Experimental protocol and results of method development in leucocytes

3.4.1 Leucocyte Feasibility experiment

As an initial experiment a pooled leucocyte pellet was assayed as described using the leucocyte method described in section 2.6.1 but substituting water or various inhibitors for NaT. The sample was assayed with no inhibitor, with 37.2 mmol/L NaT or with 1mM CBE.

Lysosomal beta-glucosidase is routinely assayed in leucocytes but the initial feasibility experiment showed that there is a measurable level of non-lysosomal beta-glucosidase activity in leucocytes i.e. that remaining in the presence of CBE (**Table 3.4**). The addition of sodium taurocholate led to a significant 126% increase in activity (P<.01) whereas the addition of CBE led to a 61% decrease (P<.01) indicating that GBA1 is the main source of beta-glucosidase activity in leucocytes.

Table 3.4 - Beta-glucosidase activities in a pooled leucocyte pellet.

Inhibitor	Activity measured	Beta-glucosidase Activity (nmol/hr/mg ptn)
None	Total inactivated	3.91 ± 0.09
NaT	Activated lysosomal	8.82 ± 0.22
1mM CBE	GBA1 inhibited	1.53 ± 0.19

3.4.2 Leucocyte GBA2 pH curve

To ascertain the optimum pH to measure non-lysosomal beta-glucosidase, the pooled leucocyte sample was assayed as previously described in the presence of CBE to inhibit GBA1. The sample was assayed in triplicate at each pH point with 10 µL of sample, 80 µL of the appropriate buffer and 10 µL of 20 mM CBE equivalent to a final concentration in the tube of 1 mM after the addition of 100 µL of substrate which was prepared in water rather than buffer. The buffers ranged from pH 2.8 to pH 8.0. Blanks were set up in duplicate for each pH as per samples but substituting water for sample.

The maximum activity in the presence of 1mM of the GBA1 inhibitor CBE was at pH 5.4-5.6 (Figure 3.10) which indicates that under these assay conditions the pH optimum of GBA2 is pH 5.4.

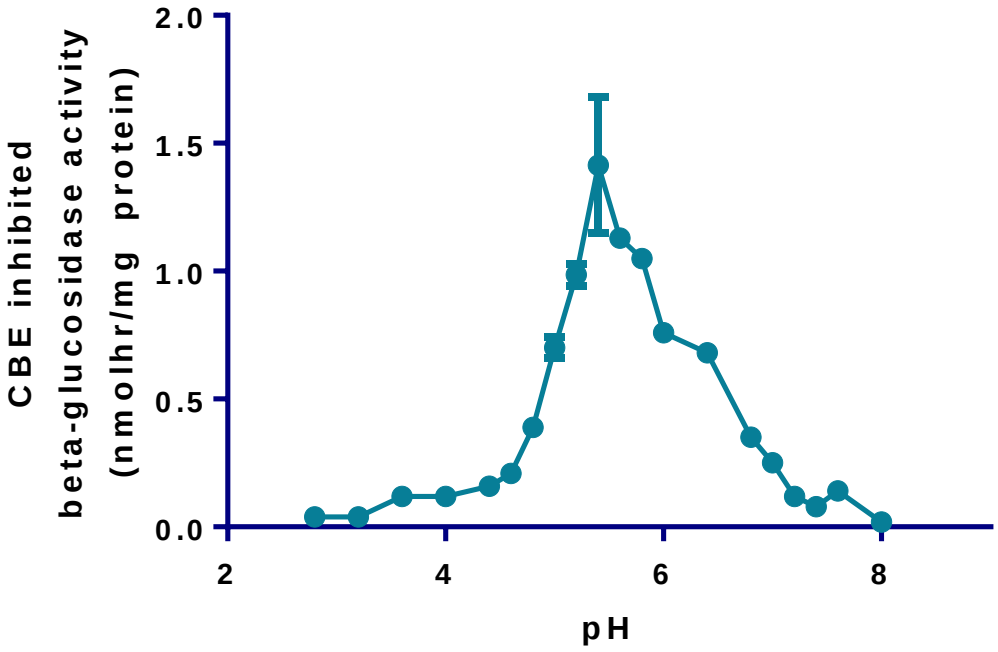

Figure 3.10 - Brain beta-glucosidase activity at various pH in the presence of, 1mM CBE. The maximum activity was at pH 5.4-5.6.

3.4.3 CBE inhibition of leucocyte beta-glucosidase activity

To ascertain the optimum concentration of CBE to inhibit lysosomal beta-glucosidase and the time required for inhibition to happen, a pooled leucocyte pellet was assayed as described previously with a range of CBE concentrations from 0-5mM. CBE or water was added to the samples which were left to incubate on ice for times varying from 5-60 minutes before assay. Blanks were set up for each CBE concentration and incubation time as per the samples but with the addition of water instead of sample.

Increasing concentrations of CBE led to increasing inhibition of beta- glucosidase activity (Figure 3.11). The level of decrease in activity was greater as the incubation time with CBE increased indicating that unlike brain there is a time-dependent inhibition with CBE. After 60 minutes of pre-incubation with 5 mM CBE, there was a 77% decrease in activity which would indicate that GBA1 accounts for most of the beta-glucosidase activity in leucocytes.

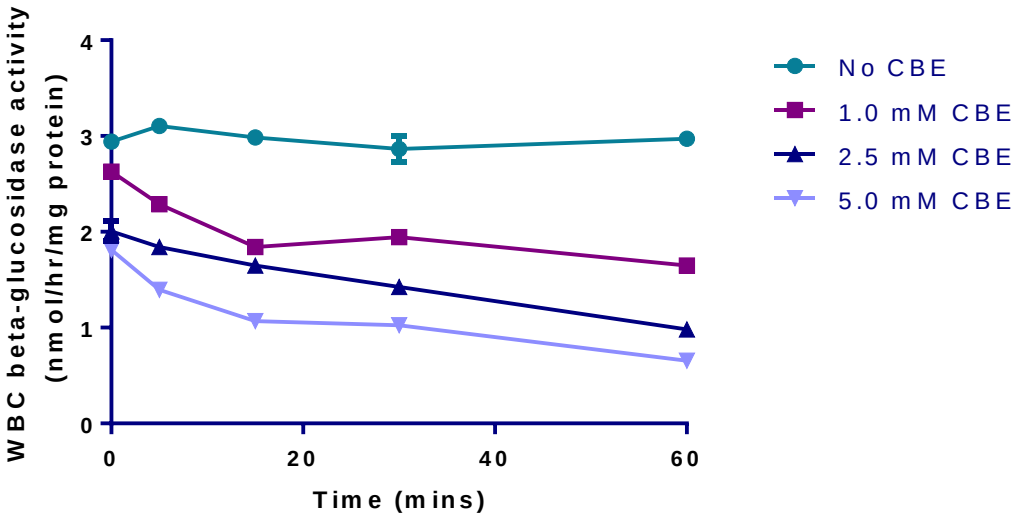

Figure 3.11 - Leucocyte beta-glucosidase activities with varying concentrations of CBE.

3.4.4 NBDNJ inhibition in leucocytes in the presence of CBE

Two sets of tubes were set up in triplicate with 10 μL of the pooled leucocyte sample and NBDNJ varying from 0-3 μM in the tube. One set contained of tubes contained no CBE while the other set contained 1.0 mM. Blanks were set up for each condition with water replacing sample. Beta-glucosidase activity was measured as previously described.

There was a 64% decrease in beta-glucosidase activity when assayed with 2.5 μM NBDNJ in the absence of CBE. Increasing NBDNJ had a negligible effect (**Figure 3.12**). In contrast to the previous experiment this would indicate that GBA1 accounts for most of the beta-glucosidase activity in leucocytes.

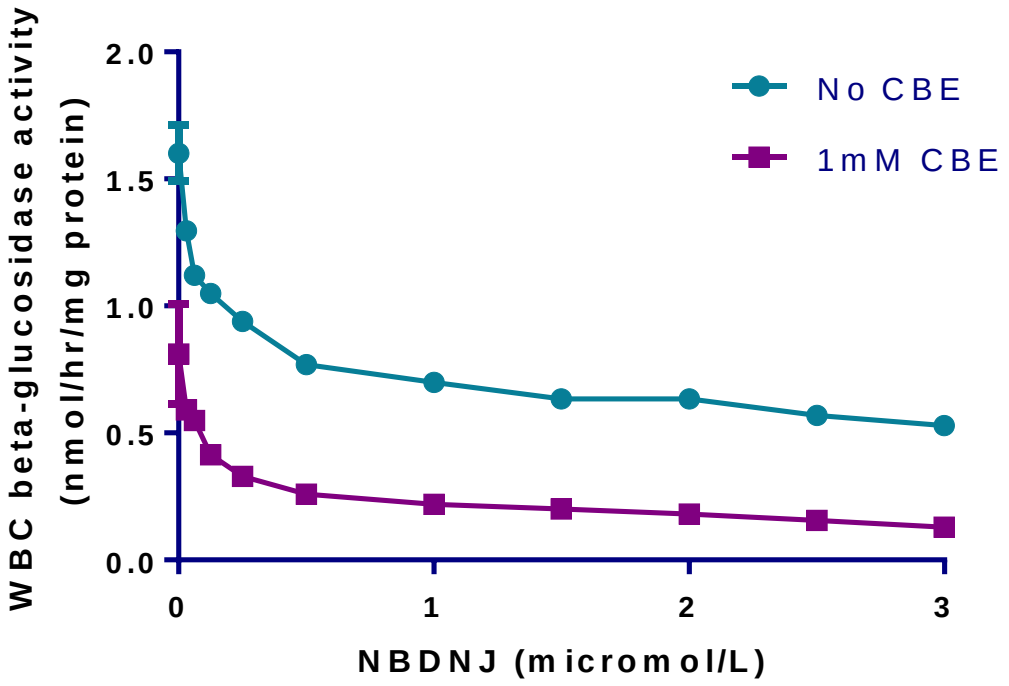

Figure 3.12 - Brain beta-glucosidase activities with varying concentrations of NBDNJ in the presence of no or 1mM mm CBE.

3.4.5 The effect of substrate concentration on leucocyte beta-glucosidase activity

To ascertain the optimum substrate concentration, a pooled leucocyte pellet was assayed in triplicate as previously with substrate ranging from 1mM to 20mM. Blanks were set up in duplicate for each substrate concentration as per samples but substituting water for sample. Each substrate concentration was assayed with either 1mM CBE or 2.5 μM NBDNJ.

Enzyme activity had reached maximum velocity by 10mM substrate. Experiments to date have used 10mM substrate and future experiments and assays will be done at this concentration (**Figure 3.13**).

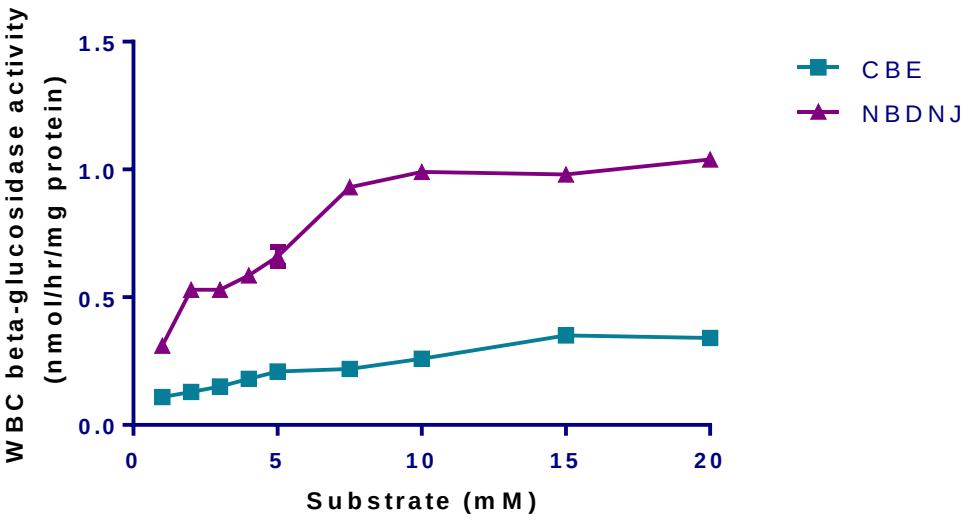

Figure 3.13 - Leucocyte beta-glucosidase activities with varying substrate concentrations in the presence of 1 mM CBE or 2.5 μM NBDNJ.

3.4.6 Summary of leucocyte GBA1 and GBA2 assays

GBA1 and GBA2 are measureable in leucocytes. Cell pellets are disrupted by sonication at amplitude 8 for 10 seconds, protein measured and the sample diluted down to 3mg/mL protein in water. 20 μL of 3 mg/mL sample is incubated with 100 μL of 5mM 4–methylumbelliferyl–β–D–glucopyranoside substrate in MV buffer pH 5.4 for +37 ^{o} C for 60 minutes before stopping with 1.0 mL 0.25 M glycine/NaOH buffer (pH 10.4) and reading the fluorescence at excitation 365 nm, emission 450 nm. GBA1 is measured by performing the assay with 80 μL 37.2 mmol/L sodium taurocholate, whereas GBA2 is measured by performing the assay with and without 2.5 μmol NBDNJ.

The assayed conditions evaluated in brain and leucocytes will be used to investigate GBA1 and GBA2 activities in plasma, cultured fibroblasts, CSF, SH-SY5Y and 1321N1 cells.

3.5 Experimental protocol and results of method development in plasma

Feasibility experiment to determine if beta-glucosidase activities are measureable in plasma

As an initial experiment, plasma was assayed as described using the fibroblast GBA1 method described section 2.6.2 but substituting water or various inhibitors for NaT. The samples were assayed in the presence of no inhibitor, 1mM CBE, 2.5 μM NBDNJ, 1mM CBE with 2.5 μM NBDNJ, and 148.8 mM sodium taurocholate. Each tube contained 20 μL of plasma, 40 μL of inhibitor or water and the volume made up to 100 μL with water. At timed intervals 100 μL of substrate (10mM in MV 5.4 buffer) was added to each tube, which was briefly vortexed before incubating at +37 ^{o} C for 60 minutes. Following the incubation, the reaction was stopped by the addition of 1.0 mL 0.25 M glycine/NaOH buffer pH 10.4 before reading on the LS55 fluorimeter (Perkin Elmer).

There was minimal fluorescence detected in any of the samples after 60 minutes incubation using 20 μL of sample.

The experiment was repeated as above but using 20 μL of sample and incubating for 20 hours. Blanks were set up for each sample and assay condition as per the tests but with the addition of the plasma which had been incubated separately at +37 ^{o} C for 20 hours, to the substrate and inhibitors after the incubation and addition of the stopping reagent.

When the experiment was repeated using 20 μL of sample and incubating for 20 hours much higher fluorescence was detected but the activity measured was still very low.

The experiment was repeated in three control samples as above but using 50 μL of sample and incubating for 22 hours.

When the experiment was repeated using 50 μL of sample and incubating for 22 hours much higher fluorescence values were detected. Incubation with CBE led to a large decrease in activity, indicating that beta-glucosidase activity in plasma is

largely GBA1. This was confirmed by the large increase in activity when incubated with Nat and the small decrease in activity seen when assayed with NBDNJ (**Table 3.5**). No further method development was performed. See section 4.4.3 for further plasma results assayed as described above.

Table 3.5 - Beta-glucosidase activities in plasma.

GBA1 was estimated to be 1745 ± 29 pmol/hr/mg while GBA2 was estimated to be 43 ± 6 pmol/hr/mg (n=3).

Inhibitor	Activity (pmol/hr/mg)
None	148 ± 18
NBDNJ	105 ± 11
NaT	1745 ± 29
CBE	40 ± 7

3.6 Experimental protocol and results of method development in cultured fibroblasts

Three control fibroblasts were prepared and beta-glucosidase assayed using the fibroblast GBA1 method described in section 2.6.2 but substituting water or various inhibitors in place of NaT where required. The samples were assayed with no inhibitor, 1.0 mM CBE, 2.5 μM NBDNJ, or 148.8 mM NaT.

Fibroblasts had the most activity total GBA activity of all tissues tested, almost 100 times that of leucocytes. There was no significant difference in the activity with and without NBDNJ. As well as negligible inhibition with NBDNJ there was a 98% inhibition with CBE indicating that it was almost all GBA1 (**Table 3.6**). GBA2 activity was estimated to be 2.8 ± 6.7 nmol/hr/mg compared to GBA1 at 1121 ± 111 nmol/hr/mg. See section 4.4.4 for further results.

Table 3.6 - Beta-glucosidase activities in control cultured fibroblasts (n=3).

Inhibitor	Activity (nmol/hr/mg)
None	306.8 ± 70.9
NBDNJ	304.0 ± 67.2
NaT	1121 ± 111
CBE	7.3 ± 0.9

3.7 Experimental protocol and results of method development in CSF

Six sets of tubes were set up in duplicate with 100 μL of CSF. One set of tubes had no inhibitor, 1.0 mM CBE, 2.5 μM NBDNJ, 1.0 mM CBE and 2.5 μM NBDNJ, 148.8 mM NaT and 148.8 mM NaT with 2.5 μM NBDNJ. Blanks were set up for each condition with water replacing sample. Beta-glucosidase activity was measured as described previously in section 2.6.2.

The activity when assayed with NaT was significantly lower compared to all other conditions ($P<0.01$) which would indicate that lysosomal beta-glucosidase is not the major beta-glucosidase in CSF. However, the addition of either CBE or NBDNJ or both did not lead to a significant decrease compared to activity when no inhibitor was used (**Table 3.7**). Method development or further assays were not pursued.

Table 3.7 - Beta-glucosidase activity in a cerebrospinal fluid.

CSF assayed with no inhibitor, 1.0 mM CBE, 2.5 μM NBDNJ, 1.0 mM CBE and 2.5 μM NBDNJ, 148.8 mM NaT.

Inhibitor	Beta-glucosidase activity (pmol/hr/mL)
None	67 ± 2
CBE	57 ± 2
NBDNJ	62 ± 1
CBE &NBDNJ	59 ± 2
NaT	22 ± 2

3.8 SH-SY5Y & 1321N1 cells

Harvested SH-SY5Y & 1321N1 cells were assayed as described previously in section 3.6. Unlike brain, GBA2 did not predominate in the cultured human brain cells comprising 17.6% of the total activity in SH-SY5Y cells and 7.1% in the 1321N1 cells (**Table 3.8**). However, both activated GBA1 (NaT) and GBA2 whether calculated as the activity remaining in the presence of CBE or as the difference in activity with and without NBDNJ, were significantly higher in the neuronal cell line compared to the astrocyte cell line. GBA1 in SH-SY5Y cells was estimated to be 256.2 ± 15 compared to 144.7 ± 8.1 nmol/hr/mg protein in 1321N1 cells. GBA2 in SH-SY5Y cells was estimated to be 8.4 ± 1.6 compared to 2.5 ± 0.1 nmol/hr/mg protein in 1321N1 cells. The levels of the lysosomal enzyme b-galactosidase (b-gal) were comparable between the two cells types at 380 ± 25 in SH-SY5Y cells and 380 ± 25 in 1321N1 cells (p = 0.128)

Table 3.8 - Beta-glucosidase activities (nmol/hr/mg protein) in a cultured SH-SY5Y and 1321N1.

Inhibitor	SH-SY5Y n=5	1321N1 n=4	Two-tailed P value
None	47.7 ± 5.8	35.1 ± 1.9	0.135
NBDNJ	39.3 ± 4.6	32.6 ± 1.8	0.290
NaT	256.2 ± 15	144.7 ± 8.1	0.002
CBE	10.6 ± 1.6	4.2 ± 0.4	0.021

Cultured microglia cells assayed at the same time had a total activity of 63.5 nmol/hr/mg which was 15.2% GBA2. (cells donated by Adrian Isaacs).

3.9 Discussion

3.9.1 Brain

The initial feasibility experiment showed that beta-glucosidase activity was measurable in brain. The addition of sodium taurocholate led to a significant 72% decrease in activity ($P<0.01$) whereas the addition of 1 mM CBE led to a smaller but still significant 28% decrease ($P<0.01$) indicating that GBA1 is not the main source of beta-glucosidase activity in brain. This was confirmed by demonstrating a large decrease in activity with 2.5 μM of NBDNJ indicating that most of the activity was GBA2. There was almost no activity left when both CBE and NBDNJ were used. Maximal GBA activity was between pH 5.4-5.6 under the four conditions studied i.e. with no inhibitor, with CBE, with NBDNJ and with NaT.

Assaying with 2.5 μM of the GBA2 inhibitor NBDNJ led to a 87.6% decrease in the measurable beta-glucosidase activity whereas increasing concentrations of the GBA1 inhibitor CBE led to greater inhibition with almost 50% decrease in activity with 5.0 mM CBE. This would indicate that one of the inhibitors is not specific.

Because of the possibility that the CBE may also be inhibiting GBA2 and the possibility that GBA activity remaining after inhibition with CBE may not just be GBA2 activity, estimation of GBA2 in brain samples should be determined by assaying samples with and without pre-incubation with 2.5 μmol NBDNJ and GBA2 calculated as the difference between the two.

3.9.2 Leucocytes

Increasing concentrations of CBE which inhibits GBA1 and longer pre-incubation times gave greater inhibition of the measureable GBA activity. The remaining non-GBA 1 activity should reflect GBA2 assuming that all the GBA1 has been inhibited and that there is no other b-glucosidase present capable of acting on the fluorescent substrate used. Increasing concentrations of NBDNJ gave greater inhibition of the measureable GBA activity. The remaining non-GBA 2 activity should reflect GBA1 assuming that all the GBA2 has been inhibited and that there is no

other b-glucosidase present capable of acting on the fluorescent substrate used. GBA1 measured this way was lower than when measured with NaT but correlated well. The maximal GBA activity in the presence of CBE i.e. GBA2 activity was at pH 5.4-5.6 which is similar the known optimum pH of 5.4 for GBA1 when measured with NaT. Because of the possibility that the GBA activity remaining after inhibition with CBE may not just be GBA2 activity, estimation of GBA2 in patient samples was determined by assaying samples with and without pre-incubation with 2.5 μmol NBDNJ and GBA2 calculated as the difference between the two. The assays were done at pH 5.4 to simplify the process when simultaneously measuring GBA1 with NaT. 60 μg protein was used for assay.

Although, GBA2 was identified as being CBE-insensitive it has since been reported by Ridley *et al.* that GBA2 activity is inactivated in vitro by CBE in a time-dependent manner. CBE inactivated GBA2 less efficiently, due to a lower affinity for this enzyme (higher KI) and a lower rate of enzyme inactivation compared with the well characterized impact of CBE on the GBA1 [228]. Others have also shown that Zebrafish GBA2 which is functionally and pharmacologically similar to human GBA2 is susceptible to inhibition by CBE in a time-dependent manner [229].

It is notable that CBE inhibition in brain was not time dependent whereas it was in leucocytes.

3.9.3 Plasma

Because of the low levels of activity observed, the large amount of sample required and the fact that most of the activity was GBA1 further optimisation or investigation of the assay conditions was not pursued. However, these conditions were used to assay beta-glucosidase activities in plasma from 5 patients with Gaucher disease and 11 controls. The results are discussed in section 4.4.3.

3.9.4 SH-SY5Y and 1321N1 cells

Both GBA1 and GBA2 were measurable in the cultured SH-SY5Y and 1321N1 cells but unlike brain GBA2 did not predominate. This may be a limitation of using a cancer derived cell in monoculture which may not reflect what happens in the more complex environment of the brain. GBA2 activity has been found to increase more than three-fold during neuronal differentiation [230] and a sevenfold increase in GBA2 activity was observed in mature neurons with respect to undifferentiated cells, which was fourfold higher than GBA1, the main β-glucosidase in undifferentiated neurons [231]. However, the finding of higher levels of both GBA1 and GBA2 in neuronal cells than astrocyte cells is consistent with findings using *in situ* labelling with fluorescent activity based probes [232].

3.9.5 Fibroblasts

Fibroblasts had the most activity of all the tissues assayed. The majority of it was GBA1 activity. Because of the low levels of GBA2 activity further optimisation or investigation of the assay conditions was not pursued. However, these conditions were used to assay beta-glucosidase activities in leucocytes from 5 patients with Gaucher disease and 16 controls. The results are in section 4.4.4.

3.9.6 CSF

Although CSF might be a useful indicator of enzyme activities in the brain area, it is not a material we have ready access, particularly from Gaucher patients and so further investigation was not pursued. The preliminary experiment indicates that GBA2 activity might predominate.

General discussion

The pH optimum for GBA2 determined here in leucocytes and brain agrees with that found in early investigations of the non-lysosomal beta-glucosidase where it was found that the CBE-sensitive enzyme i.e. GBA1 had a pH optimum of about 4.5 compared to 5.5 for the CBE-insensitive enzyme [108]. However, others have found a pH optimum of and have assayed at pH 5.8 using the synthetic substrate with

1mM CBE [110, 233]. However, the data here indicates that CBE may be inhibiting GBA2 as well as GBA1 and so GBA2 activity was determined with and without NBDNJ. It has been shown that NBDBJ has an IC50 of 50 μM against Glucosylceramide synthase (GCS), 400 μM against GBA1 and only 0.23 μM against GBA2 [234] so although it too is not a specific inhibitor, its inhibition of GBA1 is negligible at the concentrations used here. Other have measured GBA2 at pH 5.8 after pre-incubation with 100 nM MDW933, which is suggested to be a more specific inhibitor of GBA2 for 30 min at 37°C [235]. There is little information about the K_m of GBA2 for GlcCer but the K_m against the artificial fluorogenic substrate is in the hundred micromolar range [134].

3.10 Conclusions

GBA2 is the predominant beta-glucosidase in brain and also in leucocytes but to a lesser extent than in brain.

There is little if any GBA2 activity in fibroblasts using these methods.

These methods are used in chapter four to document GBA1 and GBA2 activities in various tissues from Gaucher and/or Parkinson's disease clinical and model systems.

Chapter 4 GBA1 and GBA2 activities in clinical and models systems of Gaucher and/or Parkinson's disease.

4.1 INTRODUCTION

The role of GBA1 in Gaucher disease has been established since 1965 [49]. Gaucher disease has a very wide phenotype with poor genotype/phenotype correlation which indicates there may be modifying factors which influence the course of the disease. Additionally, it has only been since 1996 that its role in Parkinson's disease has been appreciated [153] but most GD patients do not develop PD which again indicates that there may be additional modifying factors [23, 178].

GBA2 previously thought to be a bile acid beta-glucosidase is now known to be a non-lysosomal glucosylceramidase and in recent years mutations in GBA2 have been identified in patients with ataxia [122, 123], hereditary spastic paraplegia [120, 121] and Marinesco-Sjögren syndrome [116].

The fact that both enzymes are capable of performing the same reactions raises the possibility that one may be able to compensate for the absence of the other.

The overall aim of this chapter is to investigate the interplay between GBA1 and GBA2 using the established methods for GBA1 and the methods developed for GBA1 and GBA2 in chapter 3 to investigate their activities in various tissues from patients with Gaucher disease, Parkinson's disease and in model systems using SH-SY5Y cells.

AIMS

In order to evaluate the potential interplay between GBA1 and GBA2 in GD and/or PD, a number of clinically available and model systems were utilised as follows:-

- Leucocyte GBA1 in PD/dystonia
    - To investigate GBA1 activity in leucocytes from patients with Parkinsonism and/or dystonia to determine the role or fate of the enzyme in those conditions.
    - To determine if leucocyte GBA1 activity is affected by age.
    - To determine if the treatments for PD can have an effect on leucocyte GBA1.
- Leucocyte GBA2 in Gaucher disease and PD/dystonia
    - To establish a reference interval for leucocyte GBA2.
    - To determine if leucocyte GBA2 activity is altered in GD, other storage disorders and PD and/or dystonia.
- Plasma GBA1 and GBA2 in Gaucher disease
    - To determine the usefulness of GBA1 and GBA2 measurement in plasma.
    - To investigate GBA1 and GBA2 activities in plasma from patients with GD.
- To investigate GBA1 and GBA2 in cultured fibroblasts from patients with GD.
- To investigate GBA1 and GBA2 in brain from a GD mouse model and human patients.
- To determine the effects of GBA1 inhibition on GBA2 activity and GBA2 inhibition on GBA1 activity in SH-SY5Y cells.

4.2 METHODS

4.2.1 Leucocytes and plasma

Leucocytes and plasma were isolated from lithium heparin blood samples and prepared by the method previously described in section 2.3. The samples had been collected after clinical consent for clinical investigation from patients at Great Ormond Street Hospital for Children NHS foundation Trust or The National Hospital for Neurology and Neurosurgery (NHNN), Queen Square, London. Samples were processed and stored in accordance with Royal College of Pathologists guidelines, UK. This included samples from patients with suspected and subsequently confirmed GD; patients with GD who were being monitored after been treated with either enzyme replacement therapy or bone-marrow transplant; and patients who had specific GBA1 measurement or a lysosomal neurological screen as part of the investigation of their dystonia or PD. The dystonia group includes all patients with dystonia regardless of the type. Control samples were from patients who had lysosomal enzymes measured as part of their investigations but were not found to have a deficiency of a lysosomal enzyme and who were not being investigated for either dystonia or PD.

4.2.2 Cultured fibroblasts

Fibroblasts from Gaucher patients and disease controls were cultured and harvested as previously described in section 2.12.2. The samples had been collected after clinical consent for clinical investigation from patients at Great Ormond Street Hospital for Children NHS foundation Trust. Samples were processed and stored in accordance with Royal College of Pathologists guidelines, UK. Control samples were from patients who had lysosomal enzymes measured as part of their investigations but were not found to have a deficiency of a lysosomal enzyme. GD samples were from patients with a clinical diagnosis of GD confirmed by enzyme testing and mutational analysis.

4.2.3 Mouse Brain

A conditional mouse model of GD type 2 (neuronopathic GD) has been developed that displays a marked loss of brain GBA1 activity with associated GlcCer accumulation [236]. The model was generated by the insertion of a loxP-neo-loxP (lnl) cassette into intron 8 of the GBA gene which causes a splicing defect. These gbalnl/lnl mice were then bred with keratin-14-Cre transgenic mice in which Cre recombinase expression is driven by the K14 promoter, allowing excision of the lnl cassette and restoration of normal GBA1 activity in the skin without which the model does not survive [236]. After an initial symptom free period of around 10 days, the mice develop a rapidly progressing neurological degeneration and death occurs by three weeks of age if not already sacrificed. This mouse model was the first genetically induced model for nGD paving the way for the investigation of pathogenic mechanisms and potential treatments [237]. These mice (lnl/lnl) were backcrossed with wild type CD1 mice (wt/wt).

Brains from mixed sex GBA1 deficient (lnl/lnl) mice at end-stage disease pathology (day 12) (n = 5) and age matched heterozygote (n = 9) (lnl/wt) and wild type (wt/wt) mice (n = 7) were removed and stored at −80 °C until assayed for this study. The brains were supplied by Ahad Rahim (UCL School of Pharmacy, University College London, London, UK) and Simon Waddington (UCL Institute of Child Health, University College London, London, UK). Mouse procedures and welfare were approved by the University College London Animal Welfare and Ethical Review Board (AWERB) and in accordance with project and personal licenses granted by the UK Home Office and the Animal (Scientific Procedures) Act of 1986.

4.2.4 Human brain

Using the methods developed, collaborators at UCL investigated GBA1 and GBA2 activity in control brains (n = 10), PD brains from GBA1 mutation carriers (PD+GBA1; n = 14), and sporadic PD brains (n = 14) obtained from the Queen Square Brain Bank for Neurological Disorders (London, UK) following local ethical approval. All PD cases met the UK Brain Bank Clinical Criteria for PD.

4.2.5 Control lysosomal enzyme activities

The activities of beta-galactosidase or total beta-hexosaminidase were determined as control enzymes as described in sections 2.7 and 2.8 respectively. The assay of control enzymes is to determine if any changes observed in GBA1 were due to an overall change in lysosomal content or activity and to exclude any samples that had become degraded from analysis.

4.2.6 Protein determination

Sample protein concentration was determined using the BCA protein method described in section 2.11.

4.3 Experimental protocol

4.3.1 Leucocyte GBA1 activity in patients with in PD and dystonia

The results of GBA1 assay on leucocyte samples from adult patients being investigated for dystonia or Parkinsonism, assayed as described in section 2.6.1 were analysed to identify the association of GBA1 with these conditions and the effect of these conditions on GBA1. Only samples with levels of the control enzyme beta-galactosidase within the established reference interval were included in the analysis.

4.3.2 Effect of age on Leucocyte GBA1

To ascertain whether there is an age related decrease in leucocyte GBA1, ten years of routine GBA1 results from the Enzyme Laboratory at Great Ormond Street Hospital were reviewed. Results from patients with incomplete data such as gender, DOB or date of sample collection were excluded as were any samples with GBA1 results in the Gaucher affected range of 0-2.5 nmol/hr/mg ptn. Only samples with unaffected levels of the control enzyme beta-galactosidase were included in the review.

4.3.3 Effect of L-Dopa treatment on leucocyte GBA activity

The finding of lower levels of GBA1 activity in some patients with PD or dystonia raised the possibility that the treatment for these disorders may affect the levels of enzyme activity. The most common drug used for the therapy of PD is l-3,4-dihydroxyphenylalanine (l-DOPA). The principal metabolite of l-DOPA is 3-methyldopa (3OMD)(**Figure 4.1**) [238].

To ascertain whether either l-DOPA or 3OMD could affect GBA1 activity, a “normal” pooled leucocyte pellet was prepared by combining ten leucocyte samples from patients who were not being investigated for either PD or dystonia. All of the samples selected had unaffected levels of leucocyte GBA1 and beta-galactosidase.

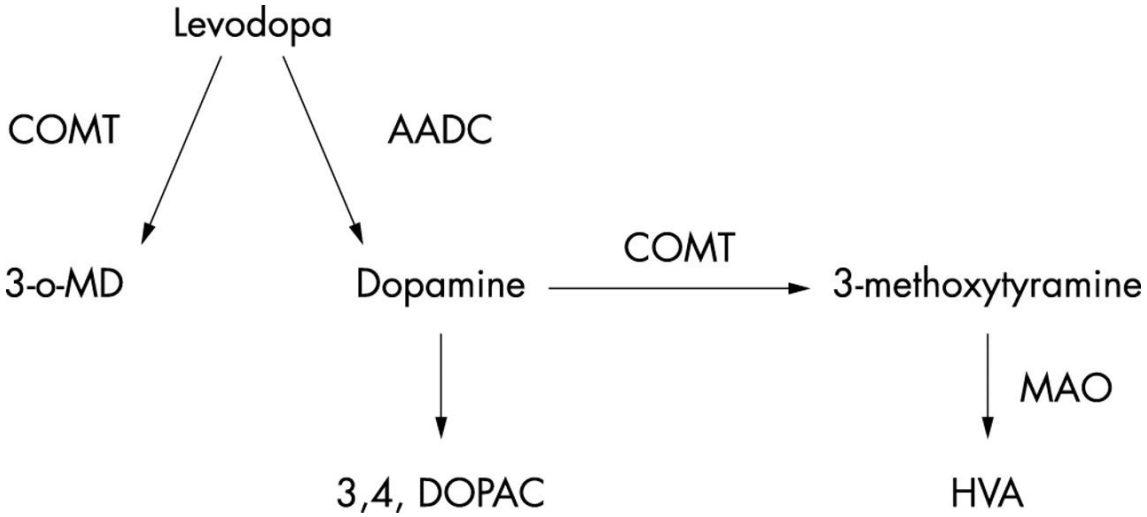

Figure 4.1 - L-DOPA metabolism (From Thanvi 2004[238])

Effect of 3-methyldopa on leucocyte GBA activity

GBA1 and beta-galactosidase were assayed in the pooled leucocyte pellet as described previously in sections 2.6.1 and 2.7.1, after being incubated with 0-60 μmol/L 3OMD at +37^{o}C for 30 minutes prior to enzyme assay to ascertain whether this principal L-DOPA metabolite could affect GBA1 activity in PD patients receiving L-DOPA. This range of 3OMD covers the reported 0-52.2 μmol/L range of the metabolite in the plasma of patients treated long term with levodopa [239].

Effect of l-DOPA on leucocyte GBA activity

GBA1 and beta-galactosidase were assayed in the pooled leucocyte pellet as described previously in sections 2.6.1 and 2.7.1, after being incubated with 0-25.9 μmol/L l-DOPA at +37 ^{o} C for 30 minutes prior to enzyme assay to ascertain whether this mainstay of PD treatment could affect b-glucosidase activity in PD patients receiving it. This range of l-DOPA covers the reported range of 2.5-14.7μmol/L (1.7 ± 1.2 mg/mL) found in the plasma of patients treated long term with it [240].

4.3.4 GBA2 in leucocytes

Using the method developed in chapter 3 (described in section 3.4.6), GBA2 activity was assayed in leucocytes from patients newly diagnosed with Gaucher disease, Gaucher disease patients on treatment, patients with other storage disorders and patients with a clinical diagnosis of Parkinsonism or dystonia. Only samples with levels of the control enzyme β-galactosidase within the established reference interval were included to exclude degraded samples.

To establish a reference interval, GBA2 results from all patients that had unaffected levels of GBA1 and did not have a diagnosis of Gaucher disease, or other storage disorder (n=111) were analysed using Analyse-it for Microsoft Excel (version 2.20) Analyse-it Software, Ltd. http://www.analyse-it.com/; 2009. Some of the control samples were assayed in the enzyme laboratory by Emmaline Cullen.

4.3.5 Plasma

Total beta-glucosidase, GBA1 and GBA2 enzyme activities were assayed as described in section 3.5, in plasma from patients with Gaucher disease and from disease controls. The control samples were from patients with unaffected levels of leucocyte GBA1. Only samples with unaffected levels of the control enzyme total beta-hexosaminidase were included.

4.3.6 Cultured fibroblasts

Total beta-glucosidase, GBA1 and GBA2 enzyme activities were assayed as described in section 3.6, in cultured fibroblasts from patients with Gaucher disease and from disease controls. The control samples were from patients who were not known or suspected to have a lysosomal storage disorder.

4.3.7 Mouse Brain GBA1 and GBA2

GBA1 and GBA2 enzyme activities were assayed as described in section 3.3.9 in brains from mice described earlier (4.2.3).

4.3.8 Human Brain

Using the methods developed, collaborators at UCL investigated GBA1 activity in the brain of 14 PD patients carrying heterozygous *GBA* mutations with characteristic PD pathology (GBA-PD), 14 patients with sporadic PD (PD) and 10 control brains. The brain regions analysed included cerebellum, frontal cortex, putamen, amygdala, and substantia nigra. The 3 groups were matched for age (control, 67.7 ± 6.0 years; PD+GBA1, 67.5 ± 2.8 years; PD, 68.9 ± 2.8 years) and post-mortem delay (control, 53.5 ± 8.1 hours; PD+GBA1, 50.5 ± 6.6 hours; PD, 41.8 ± 5.0 hours) [192].

4.3.9 Effect of GBA1 inhibition on SH-SY5Y cells

SH-SY5Y cells were cultured and harvested as described previously in section 2.12.1 but with the addition of no, 100 μM or 200 μM CBE added to the medium. Cells were harvested on day 17 and assayed as described previously in section 3.8.

4.3.10 Effect of GBA2 inhibition on SH-SY5Y cells

SH-SY5Y cells were cultured and harvested as described previously in section 2.12.1 but with the addition of no, 5mm or 10mm NBDNJ added to the medium. Cells were harvested on day 17 and assayed as described previously in section 3.8.

4.4 RESULTS

4.4.1 Leucocyte GBA1 in Parkinsonism and dystonia

GBA1 Results from 167 patients being investigated for either Parkinsonism or dystonia were reviewed. 12.6 % of this highly selective population were in the heterozygote range. 63.4% were in the overlap range between heterozygote and unaffected status so may or may not be heterozygotes.

There were 68 patients with dystonia summarised in **Table 1.1**. Although the average age of the female patients was higher than the males, the difference was not significant (p= 0.0624). Overall, 11.8% had GBA1 levels in the heterozygote range. The mean GBA1 levels were comparable between males at 7.72 ± 0.41 and 7.9 ± 0.34 in females (p= 0.7681), however more males had levels of GBA1 in the heterozygote range.

Table 4.1 - Leucocyte GBA1 results from patients being investigated for dystonia.

Numbers and percentage of patients with results in each reference range i.e. heterozygote, overlap and unaffected (above overlap range) are displayed.

Dystonia			
	All	**Male**	**Female**
n	68	34	34
Age range	17-74	18-71	17-74
Average age	43.4	39.5	47.2
Heterozygote enzyme activity (2.5-5.4 nmol/hr/mg protein)	8 (11.8%)	6 (17.6%)	2 (5.9%)
Heterozygote/unaffected overlap enzyme activity (5.4-8.9 nmol/hr/mg protein)	47 (69.1%)	21(61.8%)	26 (76.5%)
Unaffected enzyme activity (8.9-16.8 nmol/hr/mg protein)	13 (19.1%)	7 (20.6%)	6 (11.7%)

The GBA1 results are displayed in **Figure 4.2** and the control enzyme beta-galactosidase in **Figure 4.3**. There was no significant difference in the beta-galactosidase levels between the three groups i.e. patients with GBA1 in the heterozygote range, overlap range or unaffected range.

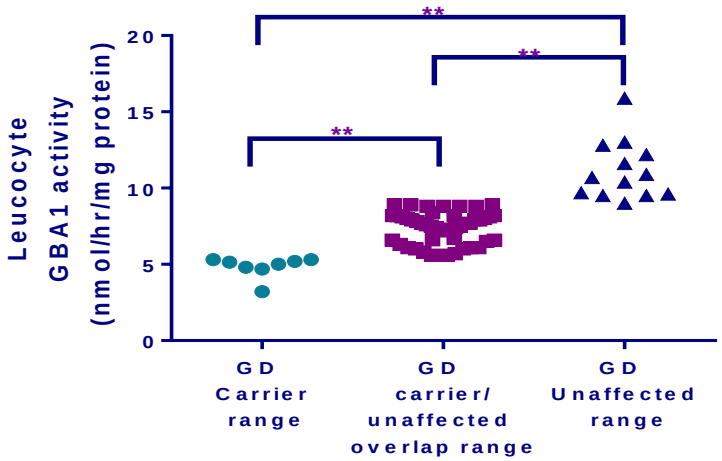

Figure 4.2 – Leucocyte GBA1 in patients with dystonia.

Data displayed as those results in the heterozygote range, those in the overlap between the heterozygote and unaffected range and those in the unaffected range (** p<0.01).

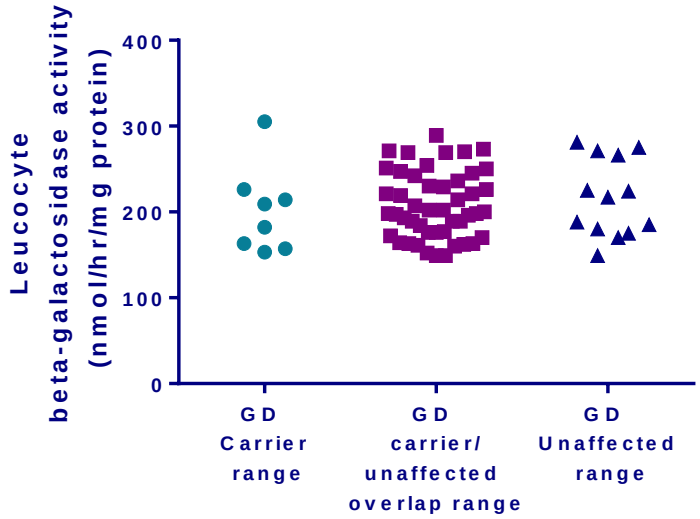

Figure 4.3 – Leucocyte beta-galactosidase in patients with dystonia.

Data displayed as those results with GBA1 in the heterozygote range, those in the overlap between the heterozygote and unaffected range and those in the unaffected range. There was no significant difference between any groups.

There were 99 patients with Parkinsonism. While there was equal numbers of male and female patients being investigated for dystonia, over 70% of the patients being investigated for Parkinsonism were male. This probably reflects the increased incidence of PD in males [241].

There was no significant difference in the average age of the males and females (p= 0.2546). Overall, 13.1% had GBA1 levels in the heterozygote range. The mean GBA1 levels were comparable between males at 8.04 ± 0.26 and 7.86 ± 0.38 in females (p= 0.7681), however in contrast to dystonia, more females had levels of GBA1 in the heterozygote range (**Table 4.2**).

Table 4.2 - Leucocyte GBA1 results from patients being investigated for Parkinsonism.

Numbers and percentage of patients with results in each reference range i.e. heterozygote, overlap and unaffected (above overlap range) are displayed.

Parkinsonism			
	All	**Male**	**Female**
n	99	71	28
Age range	29-80	29-80	41-79
Average age	54.7	53.8	56.8
Heterozygote enzyme activity (2.5-5.4 nmol/hr/mg protein)	13 (13.1%)	8 (11.3%)	5 (17.9%)
Heterozygote/unaffected overlap enzyme activity (5.4-8.9 nmol/hr/mg protein)	59 (59.6%)	43 (60.6%)	16 (57.1%)
Unaffected enzyme activity (8.9-16.8 nmol/hr/mg protein)	27 (27.3%)	20 (28.2%)	7 (25.0%)

The GBA1 results are displayed in **Figure 4.4** and the control enzyme beta-galactosidase in **Figure 4.5**. There was no significant difference in the beta-galactosidase levels between those patients with GBA1 in the heterozygote range and those in the overlap range or between those in the overlap range and the

unaffected range unaffected range. However beta-galactosidase was lower (P<.01) in the samples from those in the heterozygote at 180 ± 29 compared to those on the unaffected range at 223 ± 40 nmol/hr/mg protein.

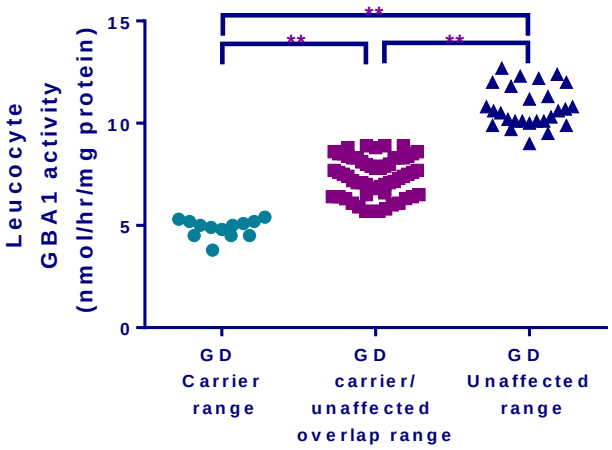

Figure 4.4 - Leucocyte GBA1 in patients with parkinsonism.

Results displayed as those in the heterozygote range, those in the overlap between the heterozygote and unaffected range and those in the unaffected range (** p<0.01).

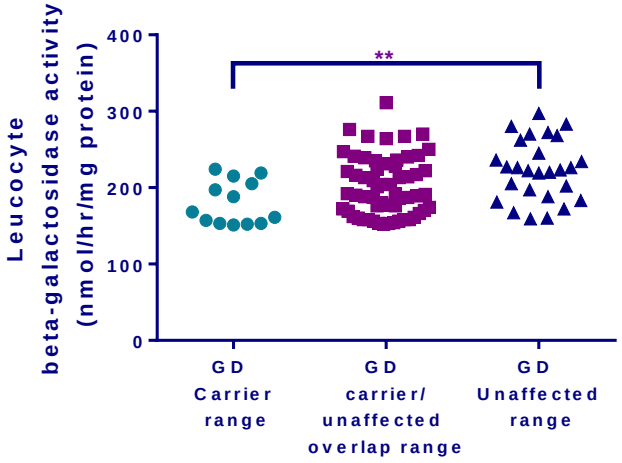

Figure 4.5 - Leucocyte beta-galactosidase in patients with dystonia.

Results displayed as those with GBA1 in the heterozygote range, those in the overlap between the heterozygote and unaffected range and those in the unaffected range (** p<0.01).

4.4.1.1 Is Leucocyte GBA1 influenced by age?

To exclude the possibility that the increased number of low GBA1 levels being observed is due to increased testing of older patients, ten years of routine leucocyte GBA1 results, assayed in the Lysosomal Laboratory at Great Ormond Street Hospital were reviewed. This includes patients being screened specifically for Gaucher disease, Parkinsonism, dystonia or other neurological symptoms. After excluding unsuitable samples or requests with incomplete data, there were 478 patient results remaining, of which there were 197 females and 281 males ranging from 3 days to 86 years of age.

While some young children had higher than average levels of GBA1 (**Figure 4.6**), there is no correlation between GBA1 activity and patient age ($R^2 = 0.0013$).

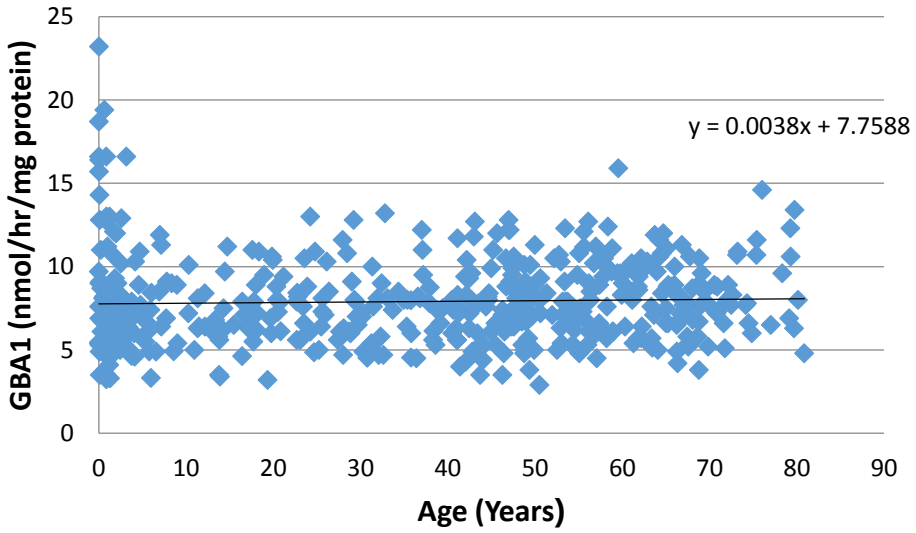

Figure 4.6 - Leucocyte GBA1 against age of patients at time of testing, n= 478, R^2 = 0.0013.

When the data was divided into age groups of fifteen year intervals (**Figure 4.7**) there was no statistical significant difference between any of the age groups.

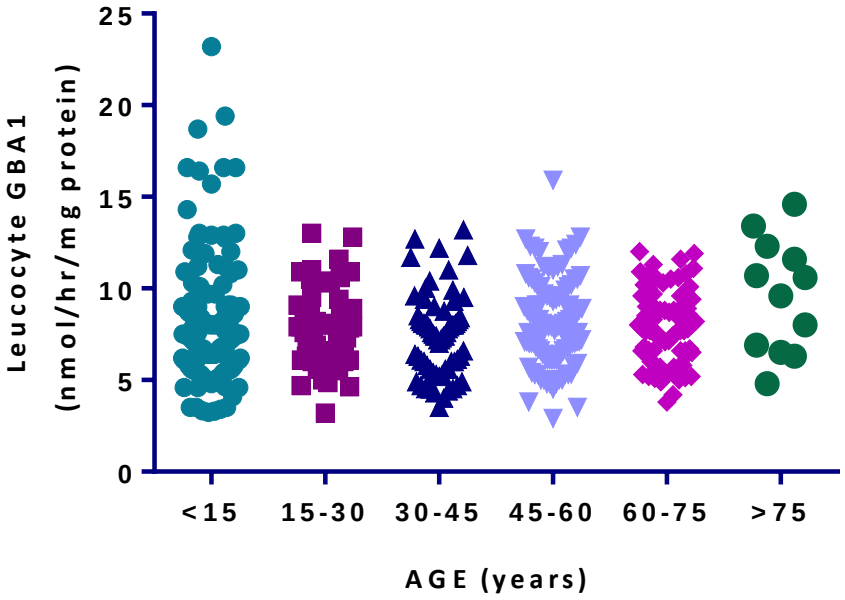

Figure 4.7 - Leucocyte GBA1 in different age groups.

To determine if there could comparative loss of GBA1 compared to other lysosomal enzymes, the ratio of GBA1 to beta-galactosidase was calculated. Again there was no evidence of an age-related decline in GBA1 (n= 478, R^2 = 0.0127).

There was no evidence of any gender differences in GBA1 values across all age groups or in the over 60s (**Table 4.3**).

Table 4.3 - GBA1 activity (nmol/hr/mg protein) in male and females.

	All ages groups		Over 60s	
	Female	**Male**	**Female**	**Male**
n	197	281	40	51
GBA1	7.66 ± 0.18	8.04 ± 0.16	7.89 ± 0.30	8.37 ± 0.34

4.4.1.2 Effect of PD treatments on leucocyte GBA activity

No effect on GBA1 or beta-galactosidase enzyme activities was observed with up to 60 µmol/L 3OMD *in vitro* (**Table 4.4**).

Table 4.4 - The effect of 3OMD on GBA1 and beta-galactosidase (B-GAL) enzyme activities (nmol/hr/mg ptn).

Enzyme	3OMD (µmol)						
	0	**10**	**20**	**30**	**40**	**50**	**60**
GBA1	9.6	9.9	9.8	9.9	9.7	9.8	9.7
B-GAL	187	187	185	186	182	188	186

No effect on leucocyte GBA1 or beta-galactosidase enzyme activity was observed with up to 25.9 µmol l-DOPA *in vitro* (**Table 4.5**).

Table 4.5 - The effect of l-DOPA on GBA1 or beta-galactosidase activities (nmol/hr/mg ptn).

Enzyme	l-DOPA (µmol)				
	0	**4.3**	**8.6**	**17.3**	**25.9**
GBA1	8.2	8.00	7.7	8.1	7.9
B-GAL	160	159	156	162	171

4.4.2 GBA2 in controls, GD, PD and other LSDs

The mean ± SEM leucocyte total beta-glucosidase activity i.e. with no inhibitor from 111 patients that did not have a diagnosis of Gaucher disease or other storage disorder was 1.79 nmol/hr/mg ptn ± 0.10. The mean ± SEM leucocyte beta-glucosidase activity assayed with NBDNJ was 0.56 nmol/hr/mg ptn ± 0.02. The mean ± SEM GBA2 activity calculated as the difference between the two, is 1.22 nmol/hr/mg ptn ± 0.09 which represents an average of 61.6% of the total beta-glucosidase activity of leucocytes.

A histogram of the GBA2 (nmol/hr/mg ptn) results with suggested reference interval using Analyse-it for Microsoft Excel (version 2.20) are displayed in **Figure 4.8**. The data is not normally distributed and suggests a reference interval of -0.997 to 3.567 nmol/hr/mg ptn.

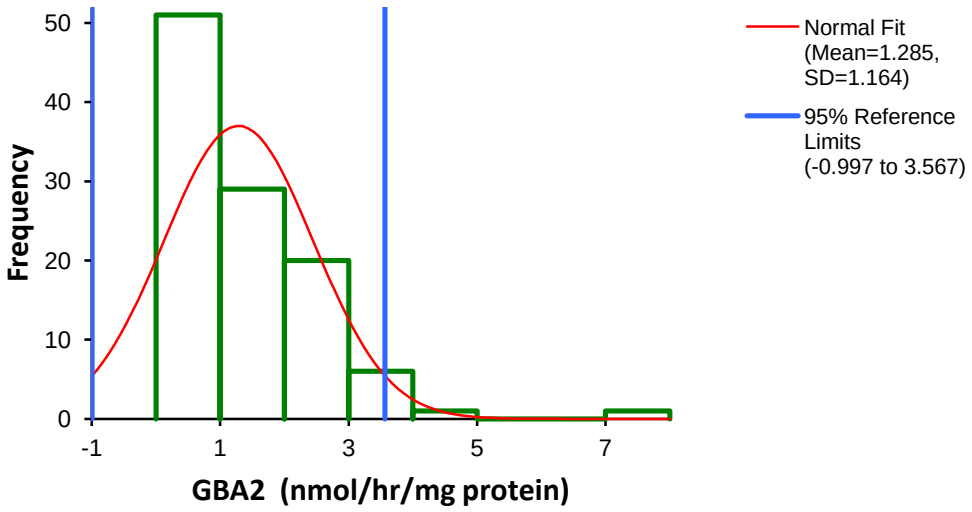

Figure 4.8 - Histogram of leucocyte GBA2 activities with suggested 95% reference limits.

Various transformations were used to normalise the data. The best fit with was log10 transformation which resulted in a 95% confidence interval of 0.124 to 3.620 nmol/hr/mg protein. Even with this transformation the data was not perfectly normalised. This could be because lower end of the “normal range” is obscured by

samples with low levels due to sample degradation. It was not possible to source many fresh leucocyte pellets and so most of the tests were done on sample remaining after routine assays.

The data for all leucocyte samples assayed is summarised in **Table 4.6** and displayed in **Figure 4.9** with results outside the established reference interval being in purple. The mean value for newly diagnosed Gaucher patients was significantly higher than the controls and patients with PD or dystonia ($p<0.05$). The mean value for other storage disorders was significantly higher than the controls and patients with PD or dystonia ($p<0.05$). All other comparisons were not significant.

Table 4.6 - Leucocyte GBA2 activity (nmol/hr/mg protein)

Leucocyte GBA2 activity in newly diagnosed GD patients, treated GD patients, patients with other storage disorders, controls and patients with either PD and or dystonia.

Patient Group	GBA2 (nmol/hr/mg protein)
New GD (n=13)	2.98 ± 0.73
Treated GD (n=12)	2.04 ± 0.58
Other Storage Disorders (n=14)	2.85 ± 0.75
Controls (n=62)	1.25 ± 0.12
PD/Dystonia (n=49)	1.32 ± 0.20
All (n=150)	1.63 ± 0.40

Of 13 newly diagnosed cases of Gaucher disease 7 (54%) were above the reference interval. In contrast only 2 of the 12 (17%) known and treated Gaucher patients had elevated GBA2 levels. Of 14 patients with a diagnosis of a different storage disorder, 5 (36%) had elevated levels of GBA2. Among the 62 samples in the "control" group, none were above the reference interval. This control group is not a "normal" control group but is composed of patients who were investigated for but were not found to have a diagnosis of a storage disorder and who were not being

investigated for parkinsonism or dystonia. Of the 49 patients being investigated for Parkinsonism or dystonia 3 (6%) had elevated levels (**Figure 4.9**).

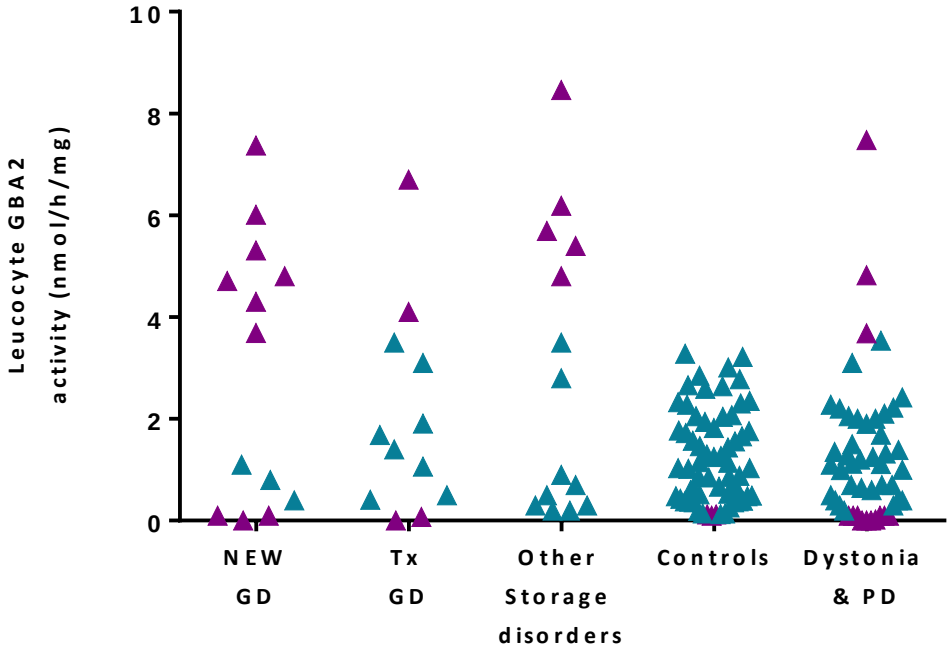

Figure 4.9 - Leucocyte GBA2 activity (nmol/hr/mg protein) in different conditions.

Results are from newly diagnosed GD patients, treated GD patients, patients with other storage disorders, controls and patients with either PD and or dystonia. Results in purple are outside the 95% confidence interval.

The mean age of the newly diagnosed GD patients was 2.98 years compared to 30.25 in the treated patients. There was no correlation between GBA2 levels and the age of the patient in either the newly diagnosed (R^2 = 0.006) or treated (R^2 = 0.027) Gaucher disease patients (**Figure 4.10**).

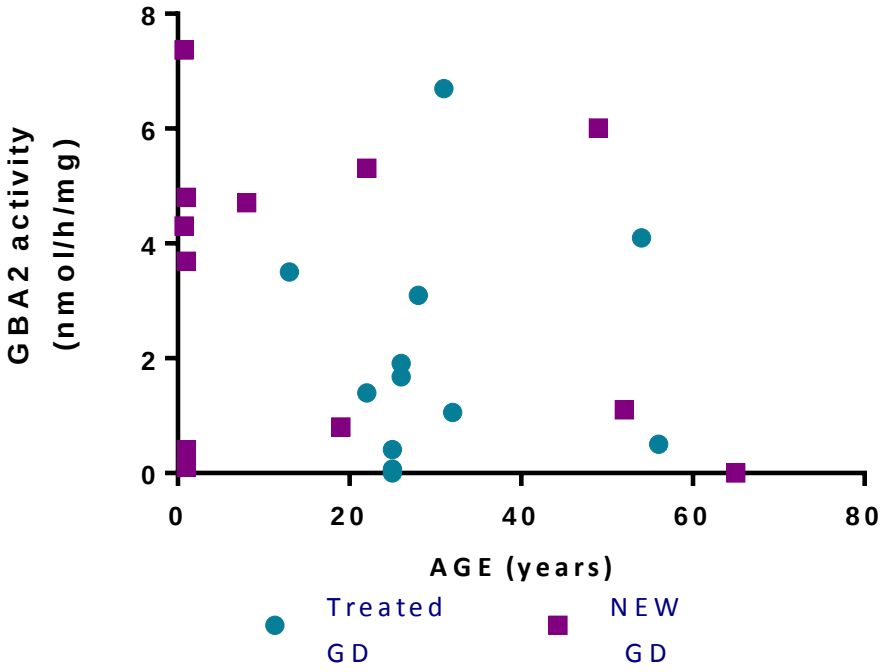

Figure 4.10 - Leucocyte GBA2 activity in newly diagnosed and treated Gaucher patients against age of patient at time of testing.

The genotype or phenotype of the majority of the newly diagnosed GD patients was not known but the second highest GBA2 value of 6.01 nmol/hr/mg protein was from a 49 year old type 1 GD patient (N370S homozygote) and the highest GBA2 of 7.37 nmol/hr/mg protein was from a type 3 GD patient (L444P homozygote).

The data for the treated patients is summarised in **Table 4.7**. Although the mean GBA2 activity was lower in GD3 patients compared to GD1 patients, the difference was not significant (p= 0.61).

Table 4.7 - GBA2 activity (nmol/hr/mg protein) in treated patients with either Gaucher disease type 1 (GD1) or type 3 (GD3).

	Gaucher Type	
	GD1 (n=7)	**GD3** (n=5)
GBA2	2.30 ± 0.89	1.66 ± 0.67
Range	0 - 6.7	0.41 -4.1

As would be expected, plasma chitotriosidase was significantly higher (p= 0.003) at 15052 ± 4321 nmol/hr/mL in newly diagnosed patients compared to 280 ± 77 nmol/hr/mL in treated patients. There was no correlation between GBA2 levels and the plasma chitotriosidase levels in new (R^2 = 0.014) or treated (R^2 = 0.059) Gaucher disease patients (**Figure 4.11**).

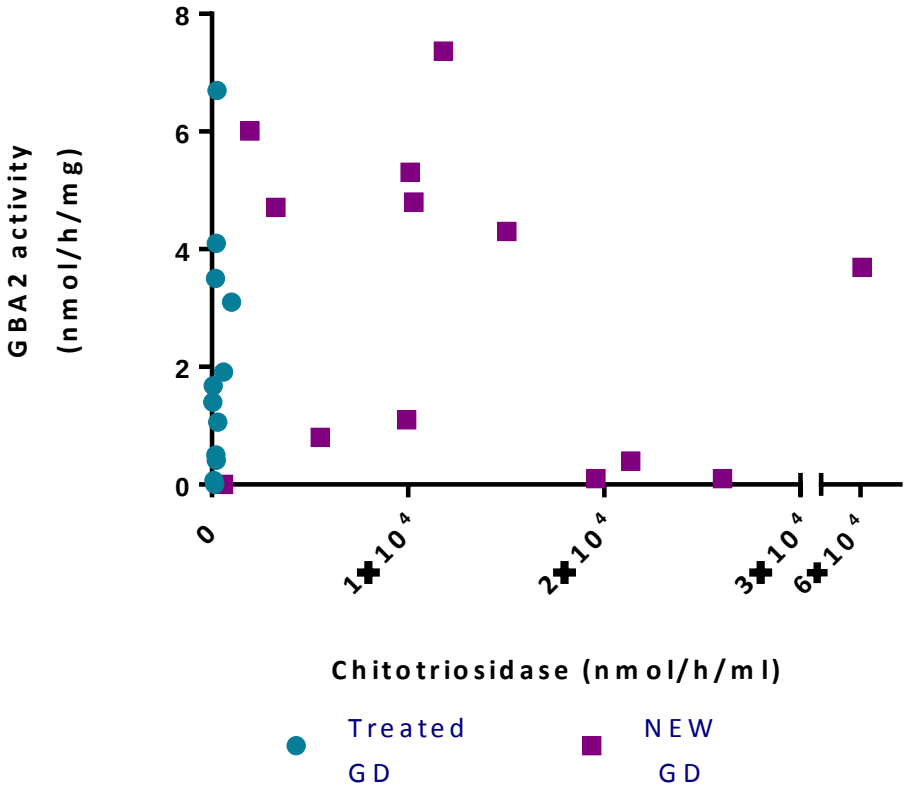

Figure 4.11 - Leucocyte GBA2 activity in newly diagnosed and treated Gaucher patients against plasma chitotriosidase.

There was no correlation between leucocyte GBA2 and GBA1 levels in new (R^2 = 0.244) or treated (R^2 = 0.004) Gaucher disease patients (**Figure 4.12**).

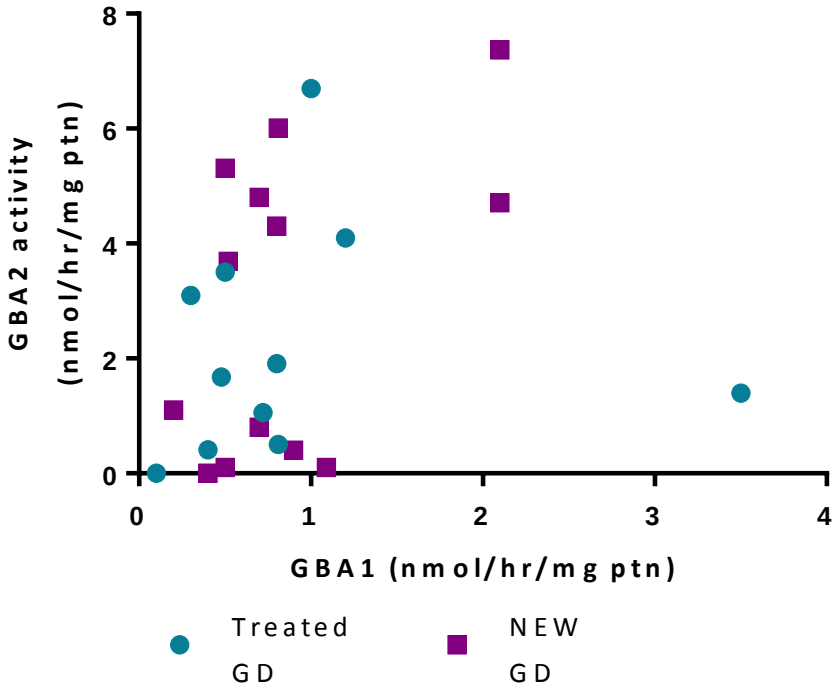

Figure 4.12 - Leucocyte GBA2 activity in newly diagnosed and treated Gaucher patients against leucocyte GBA1.

GBA2 was also assayed in 20 samples which had leucocyte GBA1 levels between 2.5 and 5.3 nmol/hr/mg protein i.e. in the carrier range above the upper limit of the GD affected reference interval and below the lower limit of the unaffected reference interval. All samples had normal levels of the control enzyme beta-galactosidase. GBA2 ranged from 0-3.54 nmol/hr/mg ptn with a mean of 0.90 ± 0.23. None had a GBA2 above the reference interval while 5 (25%) had values below the reference interval.

Other storage disorders

Of 14 patients tested with a diagnosis of a storage disorder other than Gaucher disease 5 (36%) had elevated GBA2 activity (**Table 4.8**).

Table 4.8 – Leucocyte GBA2 in patients with storage disorders other than Gaucher disease. (↑ = above upper limit of control range)

Disease	Sex	AGE (d=day, m=month, y=year)	GBA2 (nmol/hr/mg Protein)
Beta-Mannosidosis	F	4m	2.8
Mongolian blue spots ? MPS	F	1y	8.5 ↑
MPS I	F	16m	4.8 ↑
MPS I	F	10y	0.3
Pompe – GSD II	F	1d	0.2
CLN6	M	8y	6.2 ↑
Fabry	M	50y	0.3
GM2 - Tay-Sachs disease	M	18m	0.2
Hemophagocytic Lymphohistiocytosis	M	12m	5.7 ↑
Mucolipidosis	M	7d	5.4 ↑
Mucolipidosis	M	22y	0.7
MPS II	M	2y	3.5
Niemann-Pick disease A/B	M	11m	0.5
Niemann-Pick disease A/B	M	21	0.9

Samples from patients with Fabry disease, GM2 gangliosidosis (Tay-Sachs disease) and Niemann-Pick disease A/B all of which like Gaucher disease are disorders of sphingolipid metabolism had normal GBA2 enzyme activities. Samples from patients with Beta-Mannosidosis, Pompe disease (GSD II) and MPS II (Hunter disease) also had normal GBA2 levels. Of the two patients with MPS I (Hurler disease), a

lysosomal storage disorder of mucopolysaccharide metabolism, one from a 16 month old patient had an elevated GBA2 whereas the other from a 10 year old had normal level. Likewise, of the two patients with mucolipidosis, one from a 7 day old patient had an elevated level whereas the other from a 22 year old had a normal level.

Elevated GBA2 was seen in CLN6, a late infantile variant neuronal ceroid-lipofuscinosis and in Hemophagocytic Lymphohistiocytosis, a rare but potentially fatal disease of normal but overactive histiocytes and lymphocytes. The highest GBA2 observed in all the samples assayed was from a patient with a suspected but currently undiagnosed disorder who presented with blue Mongolian spots.

Low or undetectable GBA2 activities were observed in 23% of new GD patients, 17% of treated GD patients, none of the patients with other storage disorders, 5% of the controls and 20% of the PD/dystonia patients.

4.4.3 Plasma

The plasma assays were not fully developed but it was possible to measure both GBA1 and GBA2 activities. GBA1 was statistically higher in control compared to GD plasma. Although higher in GD plasma, there was no significant difference in the levels of GBA2 activity between control and Gaucher plasma (**Table 4.9**).

Table 4.9 – GBA1 and GBA2 activities in plasma from controls and patients with Gaucher disease.

Activity (nmol/h/mg)	Controls (n=11)	Gaucher (n=5) *(n=4)	Two-tailed P value
GBA1	1.46 ± 0.24	0.18 ± 0.06	<0.01
GBA2	0.04 ± 0.01	0.06 ± 0.05*	0.53

Incubation with NBDNJ led to a moderate decrease in activity indicating that the majority of GBA activity in plasma was GBA1. This was confirmed with an 11 fold increase in activity when incubated with NaT.

GBA2 accounted for 29% of the total beta-glucosidase activity in control plasma.

Although GBA1 was higher as expected in controls compared to GD plasma, one of the unaffected samples had a plasma GBA1 level comparable to the Gaucher disease samples. The reason for the low activity in an unaffected patient is unknown although pseudodeficiencies in plasma of other lysosomal enzymes have been described [242].

There was poor correlation between the plasma and leucocyte GBA1 activities (R^2 = 0.327) (**Figure 4.13**).

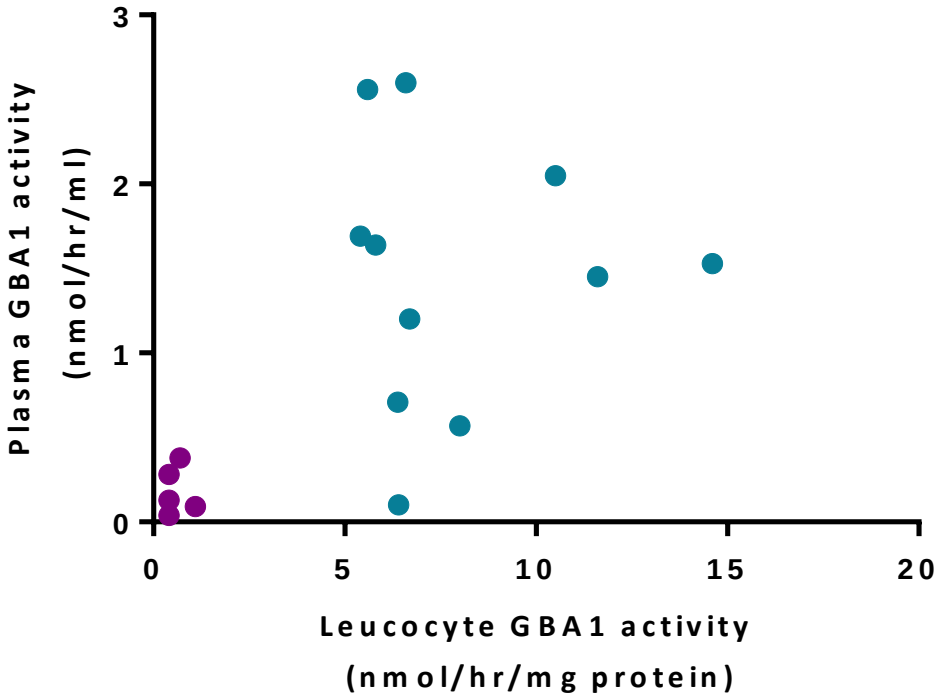

Figure 4.13 – Plasma GBA1 activity against leucocyte GBA1 from unaffected controls (blue) and patients with Gaucher disease (purple).

There was no statistically significant difference in the level of plasma GBA2 activity between control and Gaucher plasma (**Table 4.9**). However, one of the Gaucher samples had a GBA2 level that was considerably higher than any of the other Gaucher or unaffected plasma samples. Two of the unaffected patient samples and one of the GD samples had no detectable GBA2 activity (**Figure 4.14**).

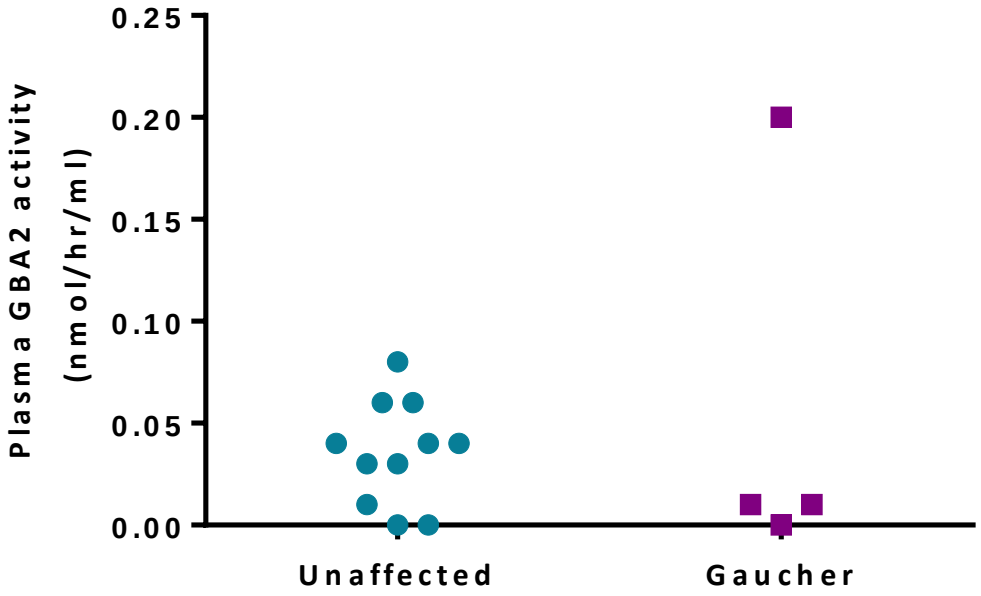

Figure 4.14 - GBA2 activity in plasma from unaffected controls (blue) and patients with Gaucher disease (purple).

There was poor correlation (R^2 =0.591) between plasma and leucocyte GBA2 (**Figure 4.15**).

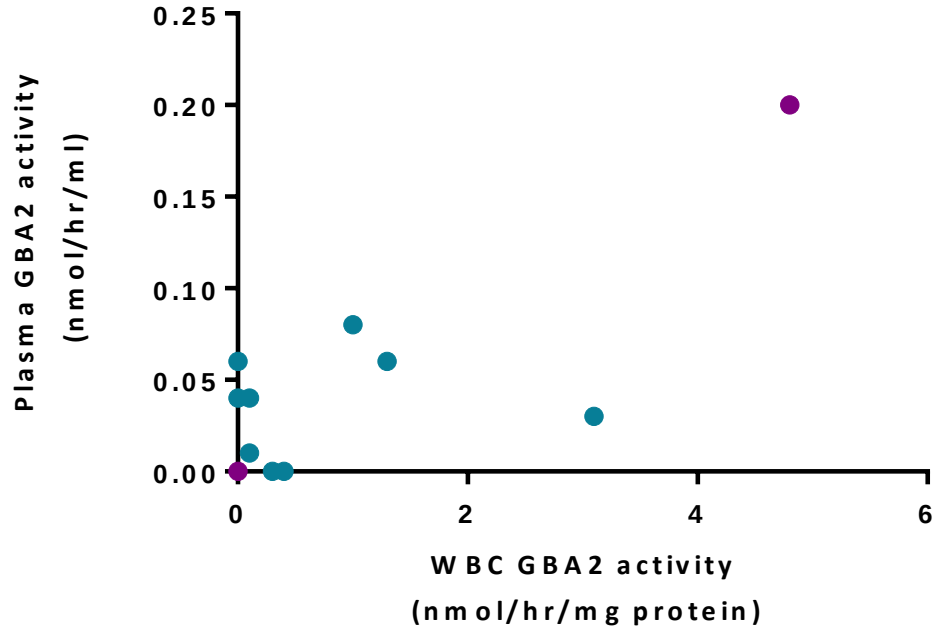

Figure 4.15 - Plasma GBA2 against leucocyte GBA2. Unaffected controls (blue) and patients with Gaucher disease (purple).

4.4.4 Cultured fibroblasts

Fibroblasts had the most total GBA activity of all tissues tested, almost 100 times that of leucocytes. There was no significant difference in the activity with and without NBDNJ in either control cells (325 ± 45 nmol/h/mg V 325 ± 45 nmol/h/mg, P=0.992) or Gaucher cells (35 ± 12 V 33 ± 11, P= 0.924). As well as negligible inhibition with NBDNJ there was a 99% inhibition with assayed in the presence of CBE indicating that most if not all beta-glucosidase activity in fibroblasts is GBA1 (**Table 4.10**).

Table 4.10 - Beta-glucosidase activities (nmol/h/mg) in cultured fibroblasts from controls and patients with Gaucher disease.

inhibitor	Controls (n=16)	Gaucher (n=5)	Two-tailed P value
None	325 ± 45	35 ± 12	0.002
NBDNJ	325 ± 45	33 ± 11	0.002
Nat	1216 ± 121	116 ± 36	<0.001
CBE	3.2 ± 0.9	0.35 ± 0.15	0.209

There was no significant difference in the measurable GBA2 levels between controls and GD cells. The mean GBA2 in controls was effectively zero (-0.6 ± 2.1. nmol/h/mg) and in GD fibroblasts (1.6 ± 0.4 nmol/h/mg). The calculated value of -0.6 nmol/h/mg is probably just a reflection of analytical imprecision.

4.4.5 GBA1 and GBA2 activity in GBA1 knockout mouse brain

This work has been published [243]. See paper in appendix.

Calculation of GBA1 and GBA2 activities in mouse brain tissue revealed that GBA2 accounts for approximately 85% of the total activity beta-glucosidase activity in wild type (wt/wt) tissue. The addition of sodium taurocholate decreased total GBA activity thereby providing further evidence to suggest that the majority of GBA activity in the brain is GBA2.

The effect of taurocholate is in contrast to that seen in tissues such as fibroblasts and leucocyte homogenates where an approximate 4 and 9 fold increase in GBA activity respectively was observed upon taurocholate addition. This is indicative of the relative predominance of GBA1 in these cell types.

As expected, GBA1 activity in brain from GBA 1 deficient (lnl/lnl) animals was markedly decreased when compared to the heterozygote (lnl/wt) or wt/wt brain **(Figure 4.16)**. For lnl/wt brains, activity was also significantly decreased when compared to wt/wt brains. Evaluation of GBA2 activity revealed a significant increase in lnl/lnl brains when compared to either lnl/wt or wt/wt brains (**Figure 4.17**). There was no significant difference in GBA2 activity between heterozygote (lnl/wt) or wt/wt brains. These results have been published (see appendix) [243].

The GBA1 assay was used again to successfully confirm the effectiveness of gene therapy in this mouse model when the long-term treated mice had a mean brain GBA1 of 21.0 ± 2.8 compared to 25.9 ± 2.6 nmol/hr/mg protein in control mice brains. There was insufficient sample to confirm if GBA2 levels had changed in response to treatment.

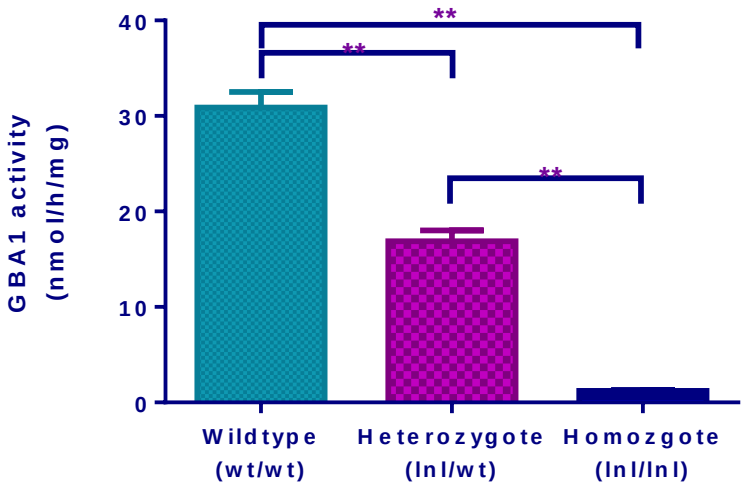

Figure 4.16 - Brain GBA1 activity in wildtype (n=7), GBA 1 heterozygotes (lnl/wt) (n=9) and GBA 1 deficient (lnl/lnl) mice (n=5) (p<0.01).**

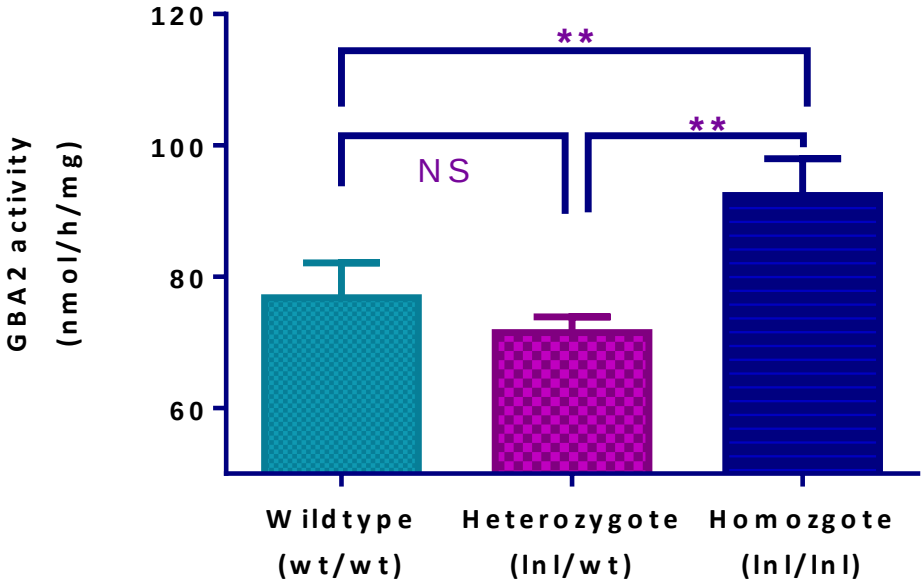

Figure 4.17 - Brain GBA2 activity in wildtype (n=7), GBA 1 heterozygotes (lnl/wt) (n=9) and GBA 1 deficient (lnl/lnl) mice (n=5)(p<0.01).**

4.4.6 Human Brain

This work has been published [192] – see appendix. GBA1 activity was significantly decreased ($p < 0.01$) in the cerebellum, putamen, amygdala and substantia nigra (SN) but not the frontal cortex of PD brains with GBA1 mutations compared to controls (**Figure 4.18**). The greatest decrease (58%) was found in SN followed by cerebellum. GBA1 activity was also significantly decreased ($p < 0.05$) but to a lesser extent in the cerebellum and substantia nigra of sporadic PD brains but not in the frontal cortex, putamen, or amygdala. It is unclear why GBA1 would be reduced in the cerebellum as it is not affected in PD [192].

GBA1 protein expression was significantly decreased in the cerebellum of both PD+GBA1 and sporadic PD brains GBA1 was significantly decreased in the putamen of PD+GBA1 brains but not sporadic PD brains. GBA1 was significantly decreased in the substantia nigra of PD+GBA1 brains and sporadic PD brains (**Figure 4.19**) [192]. No differences were found in the messenger RNA (mRNA) content of GBA indicating that alteration of the activity was not attributed to decreased expression. Other lysosomal proteins, such as beta-hexosaminidase, cathepsin D or lysosomal integral membrane protein 2 (LIMP-2), were unaffected, indicating that the loss of GBA1 activity is not due to a general reduction in lysosomal content or activity. GBA1 activity was unaffected in the amygdala of Alzheimer disease patients, suggesting that the deficiency of GBA1 seen in sporadic PD brains is not simply due to neurodegeneration [192].

The activity of GBA2 was not significantly affected in any region of either GBA-PD or PD brains compared to controls. The GBA-PD patients were heterozygotes for GD which would mean they have significant residual GBA1 activity and therefore it's unlikely that GBA2 would be increased.

GBA1 activity in control brains was highest in the putamen, followed by the amygdala, the cerebellum, frontal cortex and the lowest levels were in the substantia Nigra. GBA2 activity in control brains was highest in the cerebellum, followed by the putamen, the amygdala, the frontal cortex and the lowest levels were in the substantia Nigra [192].

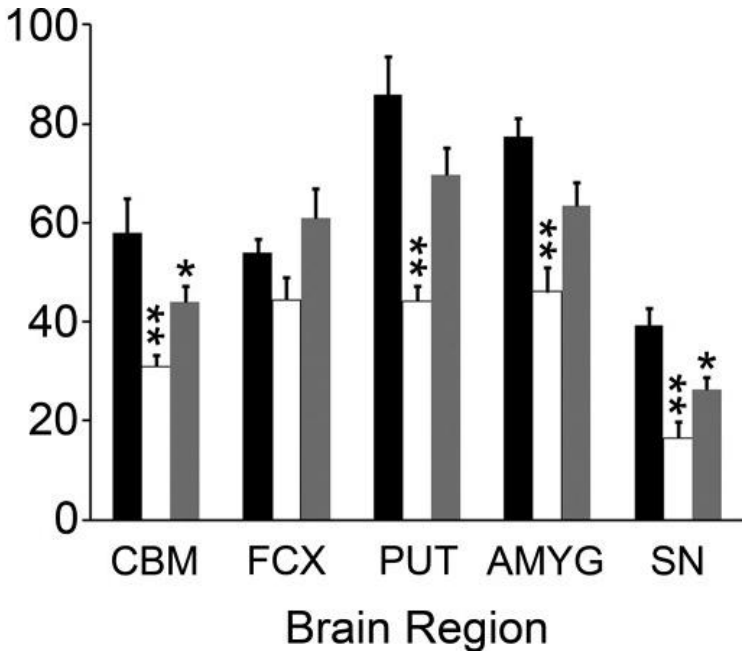

Figure 4.18 - GBA1 activity in human brain.

GBA1 activity in control brains (black bars), PD brains carrying GBA1 mutations (white bars), and sporadic PD brains (grey bars). (CBM= cerebellum, FCX = frontal cortex, PUT = putamen, AMYG = amygdala, SN = substantia Nigra). *p < 0.05 versus control, **p < 0.01 versus control (from Gegg *et al* [192]).

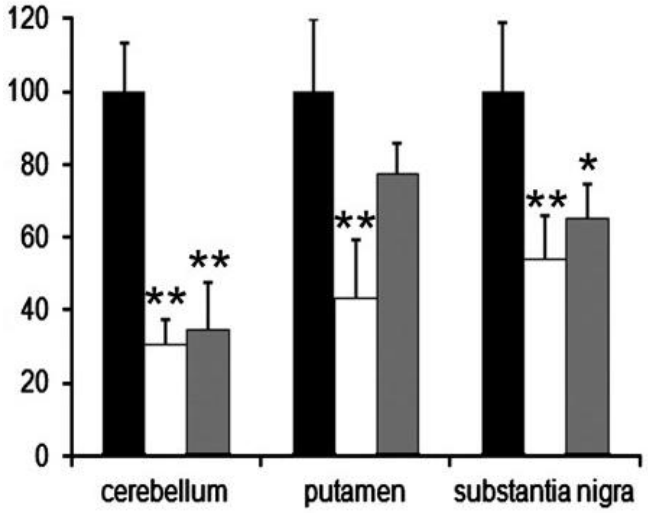

Figure 4.19 - GBA1 protein expression in human brain.

GBA1 protein expression in control brains (black bars), PD brains carrying GBA1 mutations (white bars), and sporadic PD brains (grey bars). (CBM= cerebellum, PUT = putamen, SN = substantia Nigra). *p < 0.05 versus control, **p < 0.01 versus control (from Gegg *et al* [192]).

4.4.7 GBA1 inhibition in SH-SY5Y cells.

As expected, growing SH-SY5Y cells in CBE led to a significant decrease of total beta-glucosidase and GBA1 activity (**Table 4.11**). The decrease was significant in cells harvested and tested at day 6. There was no increase in GBA2 activity in response even after 17 days (**Table 4.11**). In fact GBA2 activity at day 17 was decreased by 5.5% at 100 μmol CBE and 30.9% at 200 μmol CBE but the decrease was not significant. There was no significant change to either total beta-hexosaminidase or beta-galactosidase. There was no statistical difference for any enzyme activity between that at 100 μM CBE compared to 200 μM CBE.

Table 4.11 - Enzyme activities in SH-SY5Y cells after 17 days culture with no CBE (controls) and with 100 μM and 200 μMCBE.

Results are expressed as the mean +/- SEM and % difference with significance to control (No CBE) (n=6).

Enzyme Activity (nmol/hr/mg protein)					
	TOTAL GBA	**GBA1**	**GBA2**	**T-HEX**	**B-GAL**
No CBE (Control)	50.6 ± -2.6	246.3 ± 15.6	5.5 ± 1.5	1861 ± 104	433.5 ± 30.1
100 μM CBE	6.05 ± 0.99 - 88.0 % P<0.01	2.2 ± 0.17 - 99.1 % P<0.01	5.2 ± 0.95 - 5.5 % NS	1912 ± 175 + 2.7 % NS	382.5 ± 63.6 -11 % NS
200 μM CBE	4.55 ± 0.42 - 91.0 % P<0.01	1.6 ± 0.17 - 99.4 % P<0.01	3.8 ± 0.39 - 30.9 % NS	1846 ± 113 - 0.8 % NS	366 ± 31.6 - 15.5 % NS

4.4.8 GBA2 inhibition in SH-SY5Y cells.

Incubation with up to 10 μM NBDNJ for 17 days had no significant effect on the activities of total beta-glucosidase, GBA1, GBA2 or the control enzymes total beta-hexosaminidase or beta-galactosidase. However, the maximum decrease in GBA2 achieved at 10 μM NBDNJ was 42.3% which was not significant and may not be enough to activate any response (**Table 4.12**).

Table 4.12 - Enzyme activities in SH-SY5Y cells after 17 days with no NBDNJ (control) and with 5μm and 10 μm NBDNJ.

Results are expressed as the mean +/- SEM and % difference to control (No CBE) (n=6).

Enzyme Activity (nmol/hr/mg protein)					
	TOTAL GBA	**GBA1**	**GBA2**	**T-HEX**	**B-GAL**
No NBDNJ (control)	32.08 ± 6.7	198.9 ± 28.2	5.2 ± 1.0	1382 ± 194	337.4 ± 68.9
5 μM **NBDNJ**	24.7 ± 4.6 -23.0%	194.8 ± 19.8 -2.1%	3.2 ± 0.5 -38.5%	1176 ± 161 -14.9%	298.9 ± 62.0 -11.4%
10 μM NBDNJ	29.5 ± 6.2 -8.0%	219.6 ± 24.2 +10.4%	3.0 ± 0.6 -42.3%	1486 ± 210 +7.5%	368.3 ± 79.0 +9.1%

4.5 DISCUSSION

Leucocyte GBA1

Leucocyte GBA1 enzyme activity has been used for many years to identify patients with GD. With increased awareness of the link between GBA1 and PD it is becoming routine to measure GBA1 in patients with PD and or dystonia to exclude GD. GBA1 results in the heterozygote range can be used to identify patients for mutation analysis. With increased testing we observed a greater number of samples than expected below the well established GBA1 reference interval in patients with PD and or dystonia. It was reported by referring clinicians that a number of these patients had mutational analysis for GBA1 but were not found to have mutations. The finding that 12.6 % of those being tested had lower than normal levels of GBA1 with many not found to have GBA1 mutations suggests that some PD patients may have lower levels of enzyme due to the disease process itself. Consistent with these findings, leucocyte GBA1 activities have recently been found to be lower in GBA1 heterozygotes with PD compared asymptomatic carriers [244]. However, another group found no difference in leucocyte GBA1 activities between sporadic PD and controls [245].The low GBA1 activity in leucocytes mirrors that observed in the human brain where GBA1 enzyme activity was lower in idiopathic brains compared to controls [192]. GBA1 measured using an MS/MS assay has also been found to be lower in dried blood spot samples from patients with idiopathic PD compared to controls [246].

It has been found that GBA1 gradually diminishes with age in the substantia nigra and putamen of healthy controls. The reduction is comparable to that seen in GBA1 carriers with PD and indicates that an age dependent decrease in GBA1 activity may lower the threshold for developing PD [247]. Analysis of leucocyte GBA1 from 478 patients ranging from 3 days to 86 years of age does not suggest that leucocyte GBA1 decreases with age.

There is also no evidence that l-DOPA the most common drug used for the therapy of PD or its principal metabolite 3OMD have any effect *in vitro* on leucocyte GBA1 measurement.

It therefore seems unlikely that either patient age or PD treatment is the cause of the low GBA1 activities being observed.

GBA2 activity in human leucocytes

GBA2 activity in human leucocytes was easily measured and leucocytes would appear to be suitable sample for in vitro analysis of GBA2 activity. This has been confirmed by others [116]. GBA2 activity was found to account for approximately 61% of the total beta-glucosidase activity. Whilst this proportion is not as great as that found in brain tissue, it may indicate a metabolic role for this enzyme within leucocytes and suggest usefulness of this relatively accessible cell type for evaluating factors influencing GBA2 activity. The finding of increased leucocyte GBA2 activity, in 54% of newly diagnosed GD patients, may point to a compensatory mechanism. However, for 3 of the 13 of the new GD patients, GBA2 activity was comparable to the control group. Unfortunately, for this study it was not possible, due to the lack of available clinical information, to segregate the newly diagnosed GD patients into disease types, however there was no apparent difference in GBA2 activities between those with GD type I and type 3. Future studies are required to determine whether GD disease type and/or the magnitude of intracellular substrate accumulation influence leucocyte GBA2 activity. Individuals were identified with very low or undetectable leucocyte GBA2 activity in most groups tested, data that appears consistent with a preliminary oral communication by Yildiz who proposed that GBA2 deficiency may occur with a prevalence of approximately 5% in a control population (European Study Group on Lysosomal Diseases Meeting, 2009, Bad Honnef, Germany). The same group reported that patients with very severe GD show not just low GBA1 but also decreased GBA2 mRNA levels and no enzyme activity and elevated levels of GlcCer whereas patients with GBA2 activity had no difference in GlcCer levels compared to controls [111].

The patient in the GD group with undetectable GBA2 activity is the case report described earlier in section 1.13, which has been previously reported, to have a very mild type 1 GD associated with a severe atypical parkinsonian condition (see appendix) [193].

Leucocyte GBA2 activity was comparable between samples with GBA1 in the heterozygote range and control groups

Plasma GBA1 and GBA2

Both GBA1 and GBA2 were measurable in plasma but the activities of both are very low in comparison to leucocytes. Both required relatively large volumes and long incubation times. The level of GBA2 in comparison to total beta-glucosidase activity was very low and so leucocytes are probably a better sample to determine GBA1 and GBA2 status.

Cultured fibroblasts

Fibroblasts had the most activity total GBA activity of all tissues tested. As well as negligible inhibition with NBDNJ there was a 99% inhibition with CBE indicating that most if not all beta-glucosidase activity in fibroblasts is GBA1. There was no significant difference in the measurable GBA2 levels between controls and Gaucher cells whether calculated as the activity remaining in the presence of CBE or as the difference in activity with and without NBDNJ. These results would indicate that fibroblasts are not a suitable material for GBA2 determination. This has been confirmed by others who were unable to detect any GBA2 activity in fibroblasts [116]. This is in contrast to Aureli *et al*. who reported increased GBA2 activity in GD fibroblasts and demonstrated that this effect was particularly marked for type 1 and 2 GD cells. The increase was correlated with increased expression of GBA2 protein as evaluated by QRT-PCR [131].

Brain

As reported in chapter 3, GBA2 activity is shown here to predominate over GBA1 in brain, a finding that is supported by tissue expression studies [109, 112]. These data may imply a particular metabolic role for GBA2 in brain. The brain is the human body tissue with the highest content of GSL [134]. Our observation that brain GBA2 activity is significantly elevated in the GBA1 deficient mouse could imply the existence of a compensatory mechanism to limit substrate accumulation. Abnormally high GBA2 has also been also found in the brain of the NPC mouse [235]. The finding of increased leucocyte GBA2 activity, in 54% of newly diagnosed GD patients, may point to the existence of a similar mechanism to that occurring in the lnl/lnl mouse brain. However, due to its localisation it may have an effect on extra-lysosomal substrate accumulation but it is unlikely to affect lysosomal accumulation. The lnl/lnl mouse model utilised here displays significant brain accumulation of glucosylceramide and dramatic central nervous system (CNS) symptoms so although GBA2 levels are elevated they do not seem to be beneficial [236]. Whether glucosylceramide accumulation would be greater in the absence of an increased GBA2 activity remains to be demonstrated, however the symptoms could not be more severe without being akin to the neonatal lethal GBA1 knockout. Whether glucosylceramide accumulation directly contributes to the increase in GBA2 activity remains to be demonstrated.

GBA2 activity in lnl/wt brain was comparable to that seen in wt/wt brains. This data is also in agreement with the finding that GBA2 activity is not significantly elevated in the brain of human GBA1 heterozygotes with PD. Also, no elevated GBA2 levels were observed in leucocytes from patients with GBA1 activity in the carrier range.

The lack of response of GBA2 in heterozygotes may be due to a lack of substrate accumulation in carriers. Glucosylceramide accumulation is not reported to occur in the carriers (lnl/wt) in this mouse model [236]. And, while free glucosylsphingosine in plasma has been shown to be a highly sensitive and specific biomarker for diagnosis and monitoring in Gaucher disease, levels in heterozygotes are similar to controls [248].

SH-SY5Y cells

Inhibition of GBA1 with CBE in SH-SY5Y cells for 17 days had no effect on GBA2 activity in SH-SY5Y cells. This may be because the turnover of sphingolipids and glucosylceramide is low in cultured cells as opposed to blood cells. There is also the possibility that GBA2 was becoming inhibited by CBE. Another possibility is that GBA1 inhibition is leading to glucosylceramide and glucosylsphingosine accumulation. Glucosylsphingosine degraded by GBA2 releases sphingosine and both have been shown to inhibit GBA2 [249].

Inhibition of GBA2 with NBDNJ in SH-SY5Y cells for 17 days had no effect on GBA1 activity in SH-SY5Y cells. However, only a 42% decreased in measureable GBA1 was achieved.

4.6 CONCLUSIONS

The finding of low GBA1 activity in some patients with parkinsonism and dystonia may be secondary to factors in those conditions such as oxidative stress. This is investigated in chapter 5.

Leucocytes are a suitable sample for GBA2 measurement as is brain while plasma had very low levels of GBA2. Under the conditions used, fibroblasts had negligible if any GBA2 activity.

The findings of elevated GBA2 in the brain of a GD mouse model and in leucocytes from half of newly diagnosed GD patients would suggest that it has role in GD pathogenesis. The significance of the low or undetectable levels is currently unknown. Further work is required to understand what the significance is and whether a low or high GBA2 is preferable.

Chapter 5 EVALUATION OF THE INTERPLAY BETWEEN OXIDATIVE STRESS, MITOCHONDRIA & LYSOSOMAL FUNCTION

5.1 INTRODUCTION

When considering the pathogenesis of Parkinson's disease (PD) numerous mechanisms have been implicated for the loss of dopamine availability and degeneration of dopaminergic neurons. Amongst these, are loss of mitochondrial function, oxidative stress and impaired lysosomal GBA activity.

As regards mitochondrial function, it is of note that the brain has a particularly high energy requirement (2% of total body weight, 20% of oxygen consumption [250]. Furthermore, within the brain, dopaminergic neurons may be particularly reliant on energy metabolism due to their particularly large unmyelinated axonal arbour [251]. Evidence of compromised mitochondrial function in PD come with the description of complex 1 deficiency in the substantia nigra of patients who had died with PD [150, 252]. Support for a decreased complex I activity contributing to PD come from observations around individuals who inadvertently took MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and developed parkinsonian features [151]. Upon administration, MPTP rapidly crosses the blood brain barrier where it is metabolised to the potent complex I inhibitor MPP^+ by monoamine oxidase [152]. MPP^+ can then inhibit complex I by binding to the ubiquinone binding site [253]. This inhibition by MPP^+ in animal models has been shown to mimic the pathology of PD such as dopaminergic neuron degeneration and the formation of Lewy bodies [254, 255] . Additionally, rotenone, another complex I inhibitor, is also associated with Parkinsonism [256, 257]. In addition to loss of complex I, neuronal energy metabolism may be further compromised as a result of loss of the key mitochondrial electron carrier, ubiquinone (CoQ10). It has been demonstrated that endogenous CoQ10 levels were decreased in GD fibroblasts and treatment with CoQ10 rescued some of the subsequent mitochondrial dysfunction [258]. Additionally, several of the proteins linked to familial forms of PD, including a-synuclein, parkin and PINK are known to affect mitochondrial function and increase

oxidative stress [259]. Currently the exact mechanisms(s) responsible for the loss of mitochondrial function in idiopathic PD are not known. However, there is a growing body of evidence to implicate oxidative stress. Within the substantia nigra increased indices of oxidative stress have been documented along with accumulation of iron [260, 261] and loss of key antioxidants such as reduced glutathione (GSH) [262]. Monoamine oxidase, a key dopamine catabolic enzyme, may also contribute to the oxidative stress since a by-product of its action is generation of hydrogen peroxide [263]. The potential involvement of oxidative stress in PD comes from reports that the mitochondrial respiratory chain is susceptible to oxidative attack [264, 265]. Furthermore, complex I activity may become particularly susceptible when GSH levels are decreased [266, 267]. With regards to the oxidative stress hypothesis, it is of note that CoQ10, in addition to its role within the electron transport chain, can act as an antioxidant [268]. This property of CoQ10 therefore provides a possible explanation for the diminished availability described in PD.

In addition to compromised mitochondrial function, loss of lysosomal enzyme activity, at the level of GBA1, is also actively being investigated as a contributing factor to PD. Whilst it is established that having a mutation in GBA1, either as a heterozygote or homozygote, is a significant risk factor for developing PD [85, 197, 269] the mechanisms whereby GBA1 mutations increase risk remain unidentified. Furthermore, the majority of people with GBA1 mutations do not develop PD which indicates that there must be other factors that dictate susceptibility, e.g. GBA2 activity (see Chapter 4). Analogous to the complex I story, a specific loss of brain GBA1 activity and expression has also been reported by us and others in idiopathic PD [192, 270, 271]. This loss of activity does not appear to be confined to the brain as we have shown that leucocyte GBA1 activity is decreased in approximately 15% of patients investigated with a diagnosis of PD (section 4.3.1). A finding that has recently also been observed in blood spots from patients with idiopathic PD [246]. Currently the mechanism for this loss of GBA1 activity is not known.

5.2 AIMS

In view of the points discussed above, the aims of this chapter are to-

1. Use a neuronal cell line (SHSY5Y) to evaluate the effects of oxidative stress on cell viability in the presence of GBA1 +/- GBA2 inhibition.
2. Document the effect of oxidative stress on neuronal GBA1 activity +/- GBA2 inhibition. Additionally assess effects on other key lysosomal enzymes related directly or indirectly to sphingolipid metabolism.
3. Determine whether neuronal GBA1 activity is affected by inhibition of complex I.
4. Ascertain whether GBA1 inhibition has any effect on neuronal CoQ10
5. Evaluate the effect of GBA1 inhibition +/- oxidative stress on neuronal mitochondrial content.

5.3 METHODS

5.3.1 Cell culture

SH-SY5Y cells were cultured as described in section 2.12.1.

5.3.2 MTT viability assay

The viability of cultured cells under different conditions was determined using an in vitro toxicology kit from Sigma based on the method developed by Mosmann in 1983 and further developed by Denizot and Lang (1986). The MTT method is simple, accurate and yields reproducible results [272, 273]. The key component is (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) or MTT. Solutions of MTT, dissolved in medium or balanced salt solutions without phenol red, are yellowish in colour. Mitochondrial dehydrogenases cleave the tetrazolium ring, yielding purple formazan crystals which are insoluble in aqueous solutions. This cleavage takes place only in living cells. The crystals are dissolved in acidified isopropanol. The resulting purple solution is spectrophotometrically measured. An increase or decrease in living cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material. Working MTT was prepared as per the kit instructions (In Vitro Toxicology Assay Kit, MTT based, Sigma catalogue number TOX1-1KT) by dissolving the vial of MTT in working tissue culture medium. Working MTT was added to the flask or well plate at a volume equivalent to 10% of the medium present. The cultures were returned to the incubator for 2 hours. After the incubation period, the cultures were removed from the incubator and the resulting formazan crystals were dissolved by adding an amount of MTT Solubilisation Solution (10% Triton X-100 plus 0.1 N HCl in anhydrous isopropanol) equal to the original culture medium volume. The cultures were gently agitated to completely dissolve the MTT formazan crystals. When the assay was done in tissue culture flasks the supernatant was removed to be read in a cuvette on a Cecil CE2041 spectrophotometer. When the cells had been cultured in well plates the absorbance was read in the well plates on the Tecan Infinite M200 platereader. The absorbance of the dissolved formazan was measured at a

wavelength of 570 nm. The background absorbance was measured at 690 nm and subtracted from the 570 nm measurement.

5.3.3 Lysosomal and GBA2 enzyme activities

The activities of GBA1, GBA2, beta-galactosidase, alpha-glucosidase and total beta-hexosaminidase were determined as described in section 2.5.

5.3.4 CoQ10

CoQ10 was extracted from samples by the use of an organic solvent. The concentration of CoQ10 was then determined by reverse phase high-performance liquid chromatography (HPLC) with an on-line UV detector. CoQ10 was detected at a wavelength of 275nm.

Sample was aliquoted into an Eppendorf tube. For leucocytes 100 µL of sample was used, for SH-SY5Y cells 200 µL sample was used. 30 µL of 2.0 µM of internal standard (Dipropoxy - CoQ_{10}) was added to each Eppendorf tube (the diluted 1/10 internal standard will give a theoretical value of `1` on the HPLC chromatogram). The sample was mixed by vortexing for 20 seconds and then subjected to two cycles of freeze/thawing using liquid nitrogen. 700 µL of 5:2 (v/v) hexane/ethanol was then added to each tube which were vigorously mixed before centrifuging at 7500 RCF for 3 minutes at room temperature. The upper hexane layer was collected and stored in a fresh Eppendorf tube on ice. The lower aqueous layer was then re-extracted with 700 µL of 5:2 (v/v) hexane/ethanol, centrifuged as before and the upper hexane layer collected and combined with the upper layer from the first extraction. The combined upper layers were then evaporated by centrifuging under vacuum for 15-20 minutes at room temp using an Eppendorf concentrator 5301.

The residue was re-suspended in 300 µL of ethanol by mixing well (vortexing for 10 seconds) before filtering directly into an autosampler vial using a 4-SF-02 (PV) filter attached to a 2mL plastic syringe. The vials were capped immediately after the addition of the filtered sample. The capped autosampler vials were placed in a HPLC rack.

A scheme of the reverse-phase HPLC system is shown in **Figure 5.1**. Sample (50µL) was injected by a Jasco Intelligent AS-950 Auto-sampler and resolved using a reverse-phase Techsphere octadecasilyl column (particle size 5µm, 4.6 x 150 mm) maintained at 25^{o}C. The mobile phase was prepared by adding 1.2 mL of 60% (v/v) perchloric acid and 7g of sodium perchlorate to 700mL of ethanol and 300 mL of methanol and mixing until the sodium perchlorate was dissolved. The flow rate was maintained at 0.7mL/minute by a Jasco PU-980 Intelligent HPLC Pump. The autosampler was programmed to inject 50µL of each sample onto the column allowing a run time of 20 minutes with 5 flushes between each injection. Following separation by the column, CoQ10 was detected by a Jasco FP-920 Intelligence UV detector at a wavelength of 275nm. Data was captured and analysed on a PC with Azur Data capture and Analysis software. Prior to the detection of samples the column was washed with 50% methanol at a flow rate of 1mL/min for 1 hour with the outlet going to waste. Then the system was washed with 100% Methanol at a flow rate of 0.7mL/min for 1 hour with the outlet going to waste. The system was then equilibrated with mobile phase at a flow rate of 0.7mL/min, column temperature 25^{o}C.

After assay, the chromatograms were inspected to check the peak was well defined and had been correctly assigned by the integrator. See **Figure 5.2** for an example of a chromatogram. Protein concentration was determined using the BCA method previously described. The coenzyme Q10 result (pmol/mL) calculated automatically by the Azur software was adjusted where required to take account of the different amount of sample used and then divided by the protein result (mg/mL) to obtain final coenzyme Q10 result (pmol/mg).

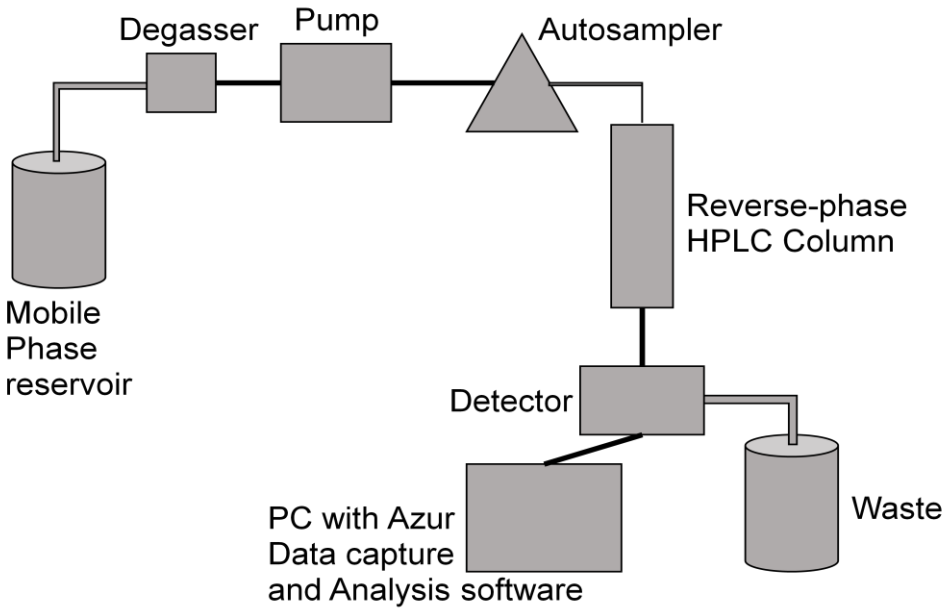

Figure 5.1- Schematic of reverse-phase HPLC apparatus used to determine CoQ10

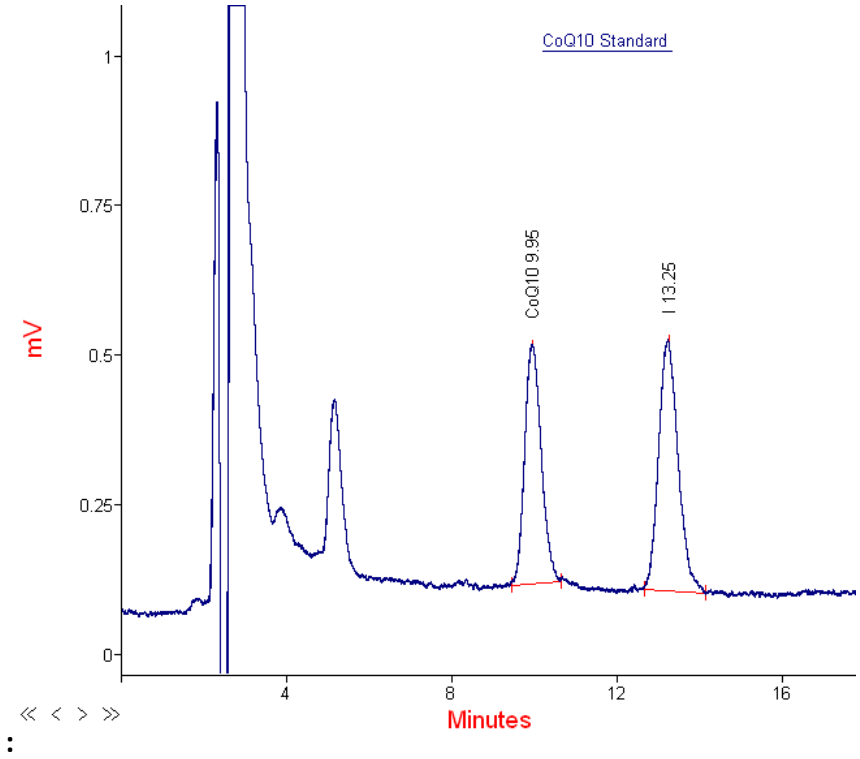

Figure 5.2 - Standard chromatogram showing CoQ10 and internal standard (I) peaks

5.3.5 Citrate synthase

Citrate synthase was determined spectrophotometrically on a Unikon 941 Plus spectrophotometer using the method of Shepherd and Garland [217] described in section 2.10.

5.3.6 Protein determination

Sample protein concentration was determined using the BCA protein method described in section 2.11.

5.4 Experimental protocol

5.4.1 The effect of H_2O_2 on cell viability

To ascertain the effect of H_2O_2 on cell viability, SH-SY5Y cells were exposed to H_2O_2 for 24 hours by the addition of H_2O_2 diluted in DMEM/F-12 to working culture medium i.e. DMEM/F-12 supplemented with 100mL/L fetal bovine serum (FBS) and 5 mmol/L L-glutamine to give a final concentration of H_2O_2 in the tissue culture flask ranging from 0.1 to 1.0 mM. The control-cultured cells were incubated with working culture medium.

5.4.2 Effect of Hydrogen Peroxide Exposure on Neuronal Lysosomal enzyme activity with and without the additional effects of inhibition of GBA1, GBA2 or both.

SH-SY5Y cells were cultured as described in section 2.12.1 for 14 days with CBE, NBDNJ or both CBE and NBDNJ. The cells were then exposed to 0, 0.1, 0.2 and 0.4 mM H_2O_2 in working medium for 24 hours before harvesting. GBA1 activity was then determined. For comparison, the activity of other key lysosomal enzymes were assessed; alpha-glucosidase, total beta-hexosaminidase and beta-galactosidase as described in section 2.5.

5.4.3 The effect of complex I inhibition using rotenone

SH-SY5Y cells were cultured as described in section 2.12.1. Cells were exposed to 0.1 or 0.25 mM rotenone for 24 hours prior to harvesting. The control-cultured cells were incubated with working culture medium. GBA1 and beta-galactosidase (control enzyme) were assayed as described in section 2.5.

5.4.4 The effect of GBA1 inhibition on CoQ10 levels

SH-SY5Y cells were cultured as described in section 2.12.1. Cells were exposed to 100 or 200 mM CBE for 12 days prior to harvesting. The control-cultured cells were incubated with working culture medium. CoQ10 was measured using HPLC as described in section 2.10. GBA1, measured to confirm inhibition and beta-galactosidase, measured as a control enzyme were assayed as described in 2.5.

5.4.5 CoQ10 measurement in leucocytes

CoQ10 was measured as described in section 5.3.4, in leucocyte samples from four patients with GD and from four controls were assayed.

5.4.6 The effect of GBA1 inhibition on Citrate synthase activity after exposure to H_2O_2.

SH-SY5Y cells were cultured as previously described and then treated with H_2O_2 at 0.1, 0.2, mM for 24 hours after 14 days pre-treatment with CBE. Citrate synthase was measured as described earlier (5.3.5).

5.5 RESULTS

5.5.1 The effect of H_2O_2 on cell viability

A dose dependent reduction in cell viability was observed with Increasing H_2O_2 concentrations (Figure 5.1). This was expected, given the known toxic effect of hydrogen peroxide, and supports the ability of the MTT assay to assess cell viability [272].

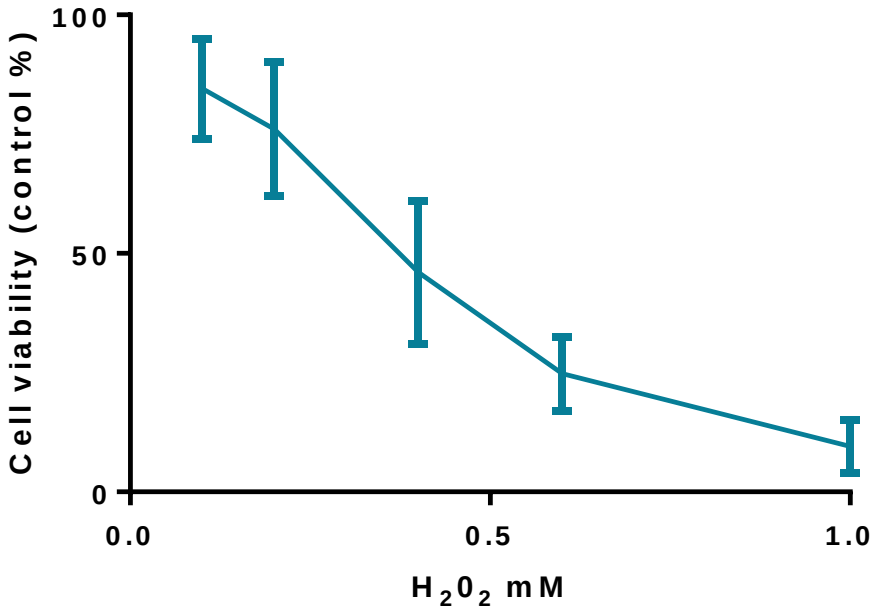

Figure 5.3 - Effect of H_2O_2 on cell viability in SHSY5Y cultured cells.

SH-SY5Y cells were treated with H_2O_2 at 0.1, 0.2, 0.4, 0.6 and 1.0mM for 24 hours. Cell viability was assessed using the MTT assay and presented as percentage of untreated (0mM H2O2) control cells (n=3 independent experiments).

The effect of H_20_2 on cell viability in the presence of GBA1 +/- GBA2 inhibition.

In view of the marked loss of viability occurring at 1.0 mM experiments with GBA1 and/or GBA2 inhibition were performed with concentrations of H_2O_2 ranging from 0.1 to 0.4 Mm (Figure 5.4). Reduction in cell viability was again observed with increasing H_2O_2 concentrations. However, there was no significant difference in the level of response to H_2O_2 when GBA1, GBA2 or both enzymes were inhibited, i.e. when compared to each other and the control cells.

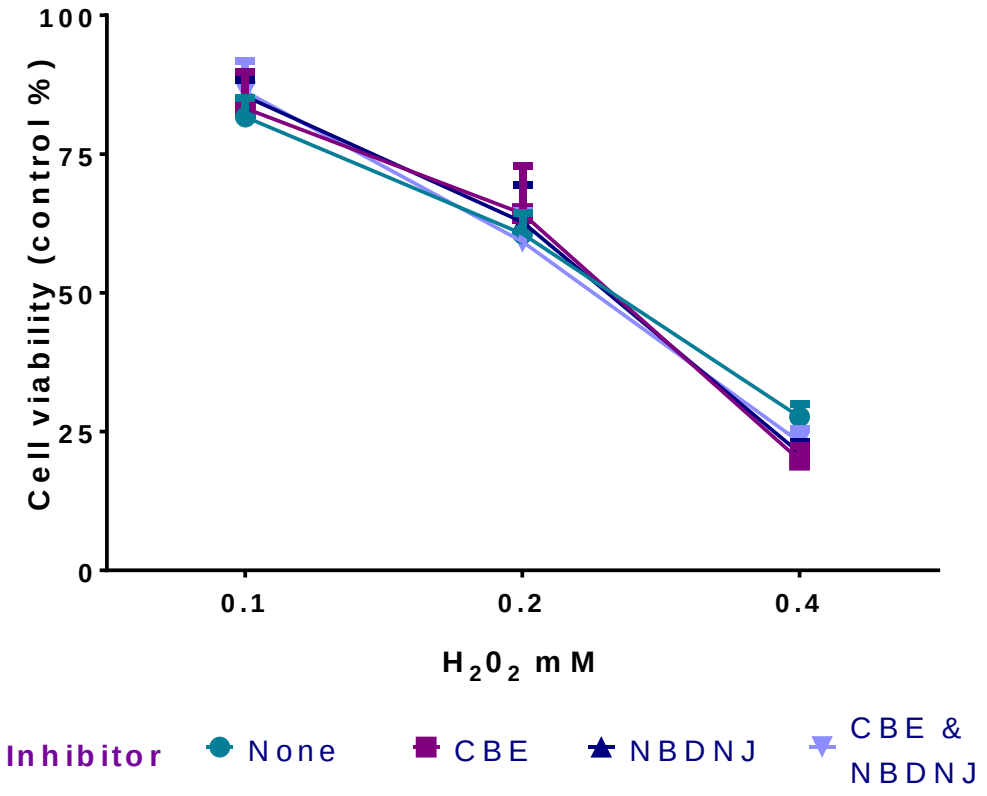

Figure 5.4 - Effects of GBA1 and/or GBA2 inhibition on the susceptibility of SHSY5Y cultured cell viability to H_2O_2.

No statistical (ANOVA) difference between any group was found (n=6).

5.5.2 Effects of Hydrogen Peroxide Exposure on Neuronal Lysosomal enzyme activity +/- the additional effects of inhibition of GBA1, GBA2 or both.

5.5.2.1 GBA1

Exposure of control SHSY5Y cells to H_20_2 resulted in an apparent dose responsive increase in GBA1 activity. However, this did not reach statistical significance **(Figure 5.5a)**. When GBA2 was inhibited, by NBDNJ, exposure of the cells to H_20_2 resulted in a significant dose response increase in GBA1 activity **(Figure 5.5a)**. In the presence of the GBA1 inhibitor, CBE, GBA1 activity was, as expected, markedly decreased compared to control cells. Exposure to H_20_2 had no significant effect on residual GBA1 activity **(Figure 5.5b)**. In the presence of both inhibitors, residual GBA1 activity was not significantly affected by H_20_2 exposure **(Figure 5.5b)**.

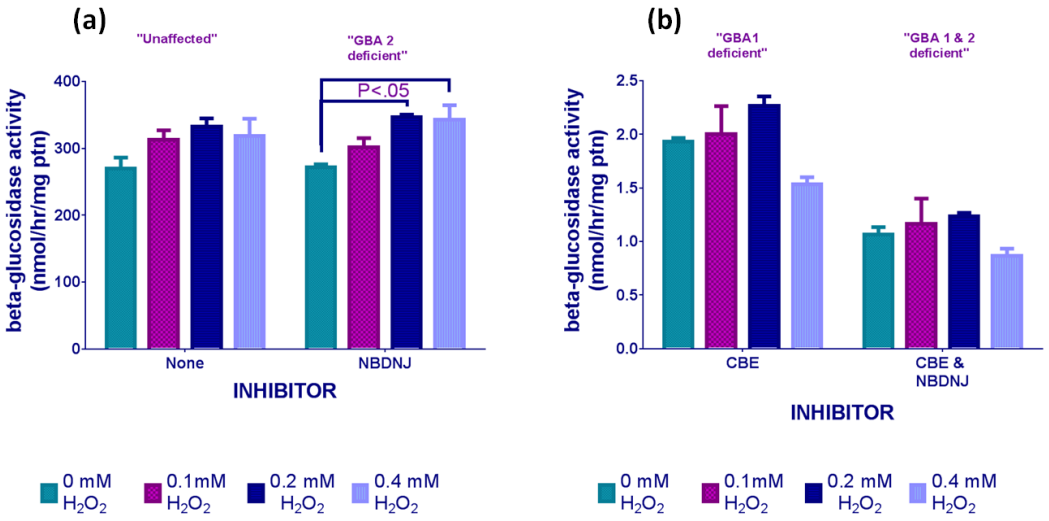

Figure 5.5 Effects of H_2O_2 on beta-glucosidase (GBA1) activity.

SH-SY5Y cells were treated with H_2O_2 at 0.1, 0.2, and 0.4, mM for 24 hours after 14 days pre-treatment with CBE, NBDNJ or both inhibitors (n=3).

5.5.2.2 Total beta-hexosaminidase

Total beta-hexosaminidase was assayed as a reference enzyme. Like GBA1, it is involved in sphingolipid metabolism. Increasing levels of H_2O_2 led to increased total beta-hexosaminidase activity in both control and inhibited cells (Figure 5.6). Activity significantly increased by 54% in control cells at 0.4 mM H_2O_2 compared to cells with no H_2O_2. The response in GBA1 inhibited and/or GBA2 inhibited cells was even greater. Activity was significantly increased by 96% in GBA1 inhibited cells, 91% in GBA2 inhibited cells and 105% when both GBA1 and GBA2 were inhibited at 0.4 mM H_2O_2 compared to cells with no H_2O_2.

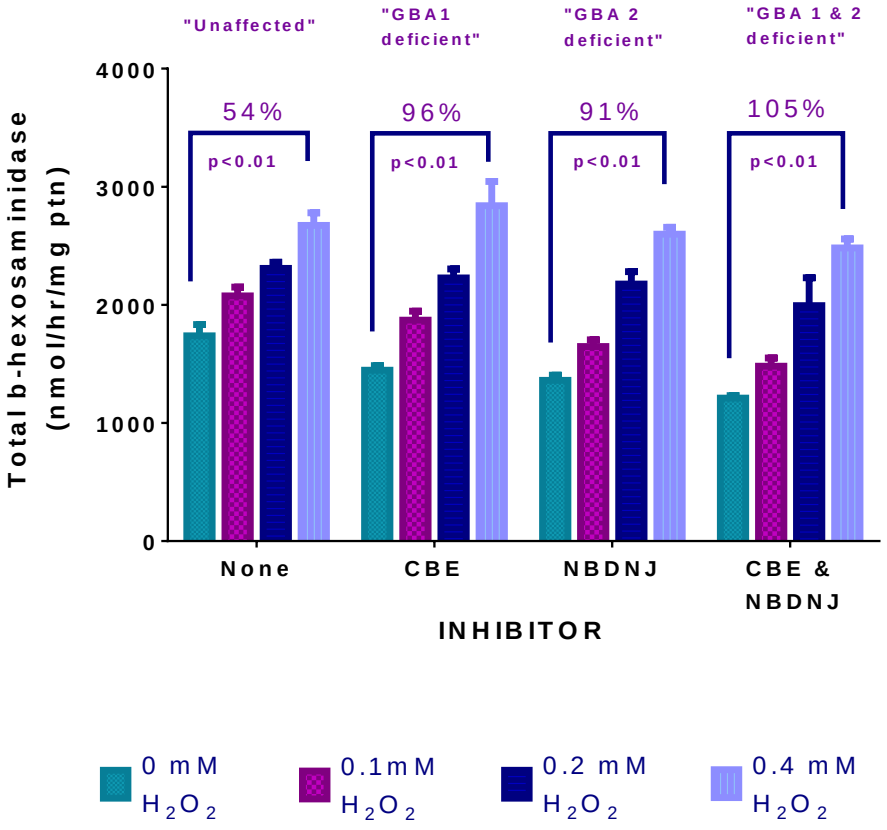

Figure 5.6 - Effects of H_2O_2 on total beta-hexosaminidase activity.

SH-SY5Y cells were treated with H_2O_2 at 0.1, 0.2, and 0.4, mM for 24 hours after 14 days pre-treatment with CBE, NBDNJ or both inhibitors (n=3).

5.5.2.3 Plasma Total beta-hexosaminidase

In light of the response of total beta-hexosaminidase to H_2O_2 observed in the GBA1 inhibited SH-SY5Y cells, and the reports in the literature of elevated total beta-hexosaminidase in GD [38, 39], activity was measured in plasma as described in section 2.8.3. The samples were from GD patients and controls as described in section 4.2.1.

Total beta-hexosaminidase was significantly higher (p= 0.031) in Gaucher plasma at 3.04 ± 1.41 (n=3) compared to 0.83 ± 0.06 in control plasma (n=12) (**Figure 5.7).** However, one GD patient had normal levels of total beta-hexosaminidase activity.

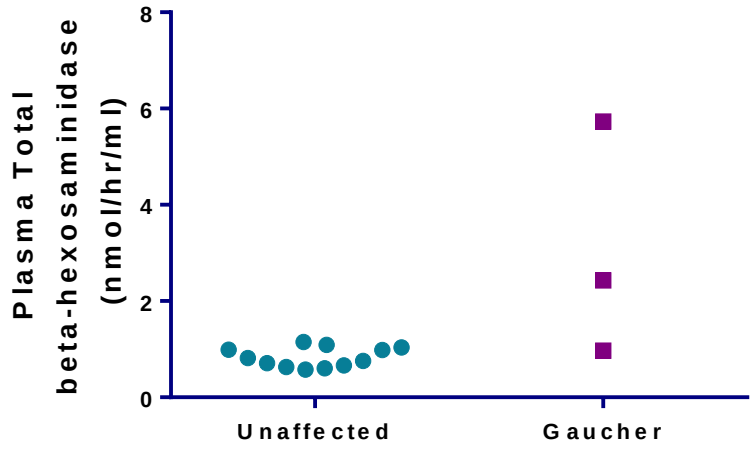

Figure 5.7 - Total beta-hexosaminidase activity in plasma from unaffected controls and patients with Gaucher disease

5.5.2.4 Alpha-glucosidase

Unlike GBA1, beta-galactosidase or total-hexosaminidase, alpha-glucosidase is not involved in sphingolipid metabolism. Both CBE and NBDNJ led to significant decreases ($P<0.01$) in alpha-glucosidase activity compared to control cells showing that neither are specific inhibitors of beta-glucosidase (**Table 5.1**).

Table 5.1 - The effect of CBE and NBDNJ inhibition on alpha glucosidase activity.

SH-SY5Y cells were treated for 14 days with CBE, NBDNJ or both inhibitors (n=3).

	Control	**GBA1 inhibited**	**GBA2 inhibited**	**GBA1 and GBA2 inhibited**
alpha-glucosidase activity (nmol/hr/mg protein)	99.2 +/- 5.7	47.1+/- 1.2	59.2 +/- 1.1	45.0 +/- 0.6

Incubation with 0.4 mM H_2O_2 led to a non-significant increase in activity of 15% in control cells and 12% in GBA1 inhibited cells. However, there was a significant increase in activity in response 0.4 mM H_2O_2 of 57% (P<.01) and 65% (P<.05) in cells where GBA2 or both GBA1 and GBA2 were inhibited respectively (**Figure 5.8**).

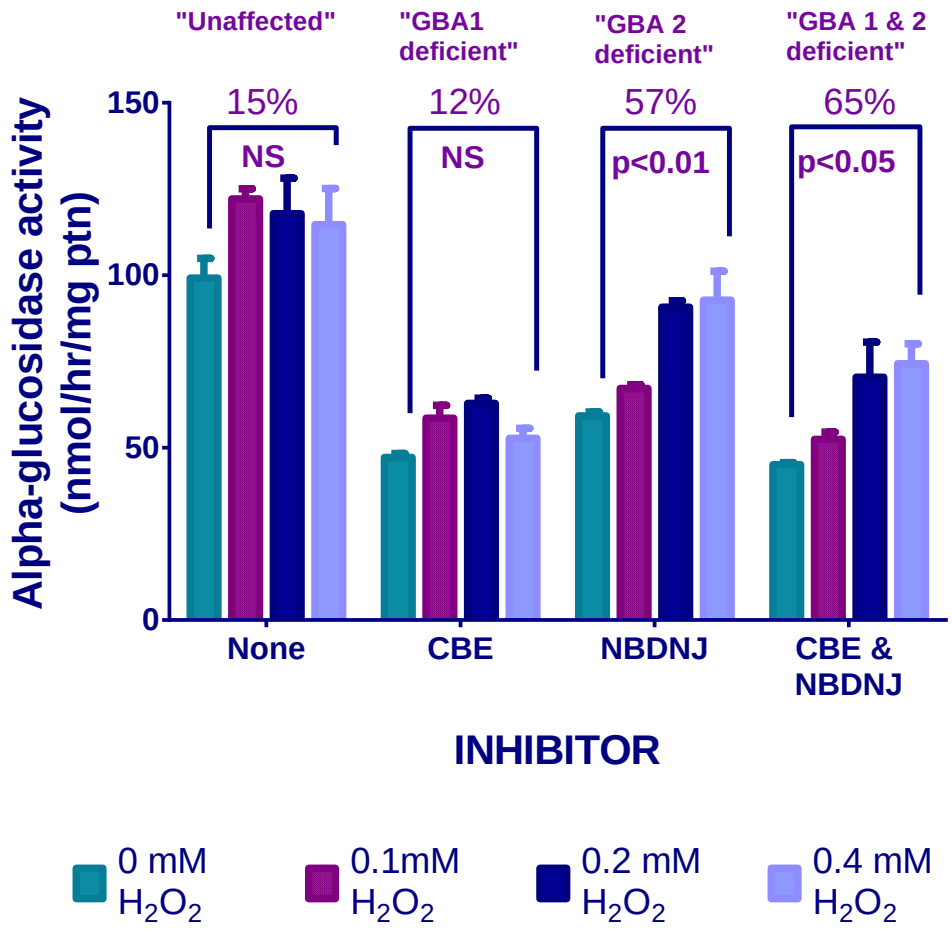

Figure 5.8 - Effects of H_2O_2 on alpha-glucosidase activity.

SH-SY5Y cells were treated with H_2O_2 at 0.1, 0.2, and 0.4, mM for 24 hours after 14 days pre-treatment with CBE, NBDNJ or both inhibitors (n=3).

5.5.2.5 Beta-galactosidase

Beta-galactosidase, like GBA and total beta-hexosaminidase, is involved in sphingolipid metabolism. However, in contrast to GBA1, there was no significant difference in the response of beta-galactosidase to H_2O_2 within or between any of the groups (**Figure 5.9**).

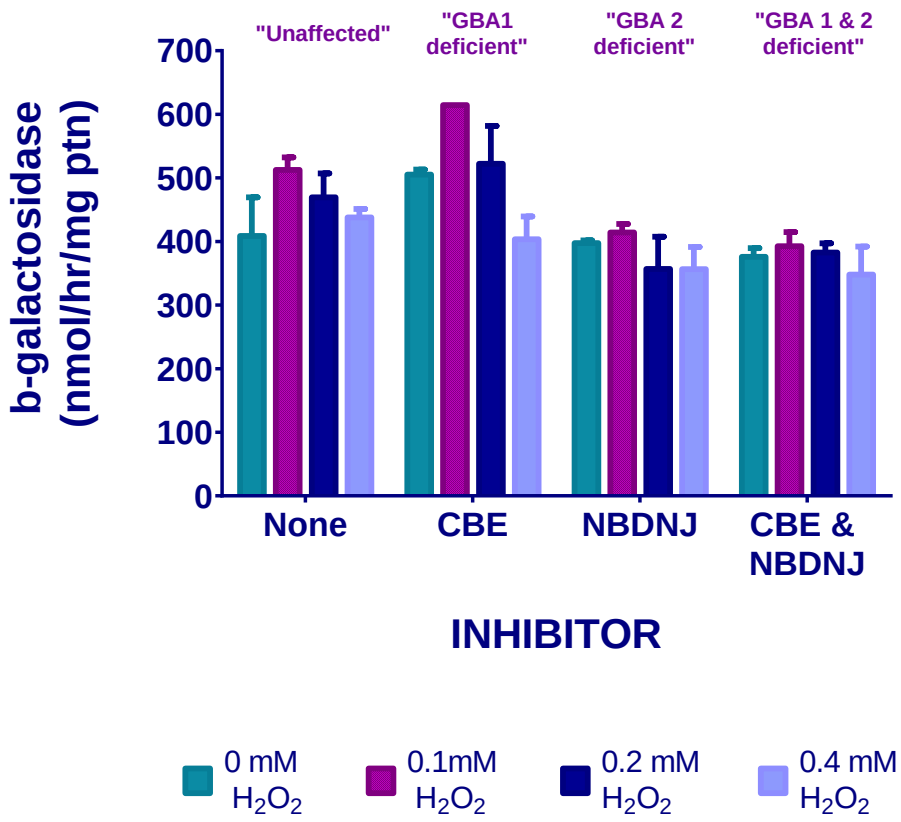

Figure 5.9 - Effects of H_2O_2 on beta-galactosidase activity.

SH-SY5Y cells were treated with H_2O_2 at 0.1, 0.2, and 0.4, mM for 24 hours after 14 days pre-treatment with CBE, NBDNJ or both inhibitors (n=3). There was no significant difference in the response of beta-galactosidase to H_2O_2 in any of the groups.

5.5.3 The effect of complex I inhibition on Lysosomal enzymes

5.5.3.1 The effect of complex I inhibition on GBA1 activity

There was a small but non-significant increase in GBA1 activity in response to complex I inhibition; GBA1 activity was 309.0 +/- 17.6 nmol/h/mg in control cells compared to 357.5 +/- 33.8 nmol/h/mg in cells with 0.1 mm rotenone and 339.8 +/- 4.4 nmol/h/mg in cells with 0.25 mm rotenone (**Figure 5.10**).

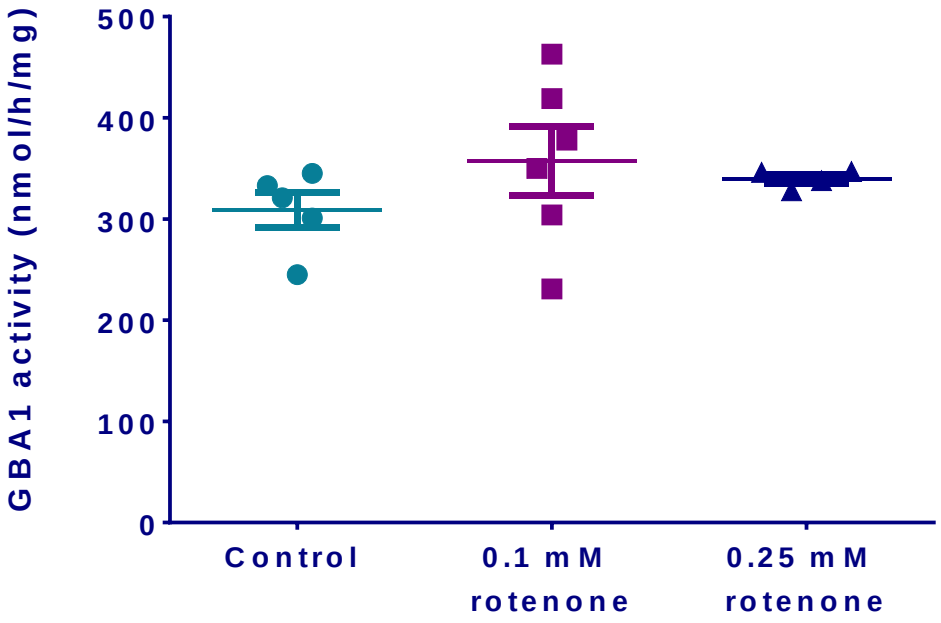

Figure 5.10 - Effects of Complex I inhibition on GBA 1 activity.

SH-SY5Y were exposed to 0.1 or 0.25 mM rotenone for 24 hours prior to harvesting. The control-cultured cells were incubated with working culture medium (n=5). No significant difference was observed between the three conditions.

5.5.3.2 The effect of complex I inhibition on beta-galactosidase activity

Beta-galactosidase activity was assayed as a control enzyme. Like GBA1, there was a small but non-significant increase in activity in response to complex I inhibition. Beta-galactosidase activity was 506 +/- 26 nmol/hr/mg protein in control cells compared to 598 +/- 56 in cells exposed to 0.1 mM rotenone and 575 +/- 7 in cells exposed to 0.25 mM rotenone (**Figure 5.11**).

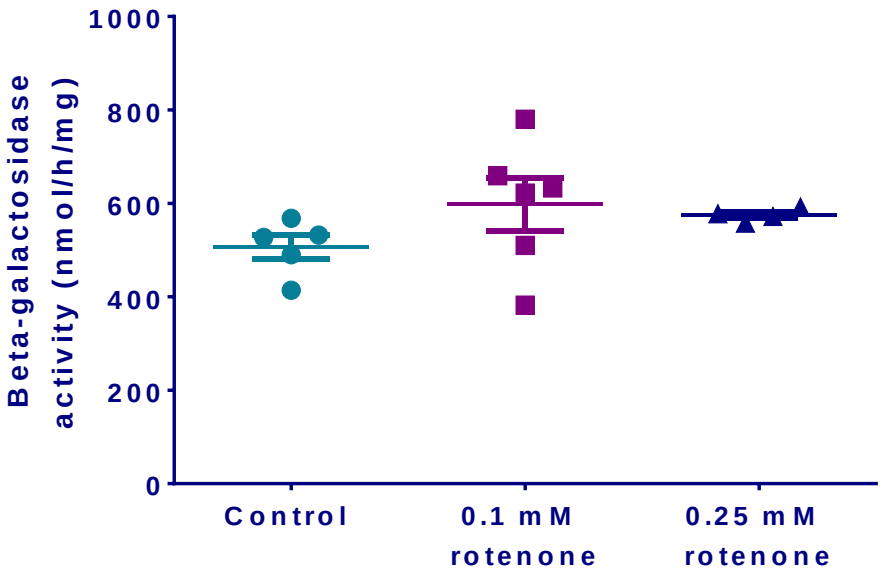

Figure 5.11 - Effects of Complex I inhibition on beta-galactosidase activity.

SH-SY5Y were exposed to 0.1 or 0.25 mM rotenone for 24 hours prior to harvesting. The control-cultured cells were incubated with working culture medium (n=5). No significant difference was observed between the three conditions.

5.5.4 Neuronal CoQ10 status following inhibition of GBA1

When compared to control cells, inhibition of GBA1 with CBE treatment had no significant effect upon SH-SY5Y CoQ10 levels (n=6) (**Figure 5.12**).

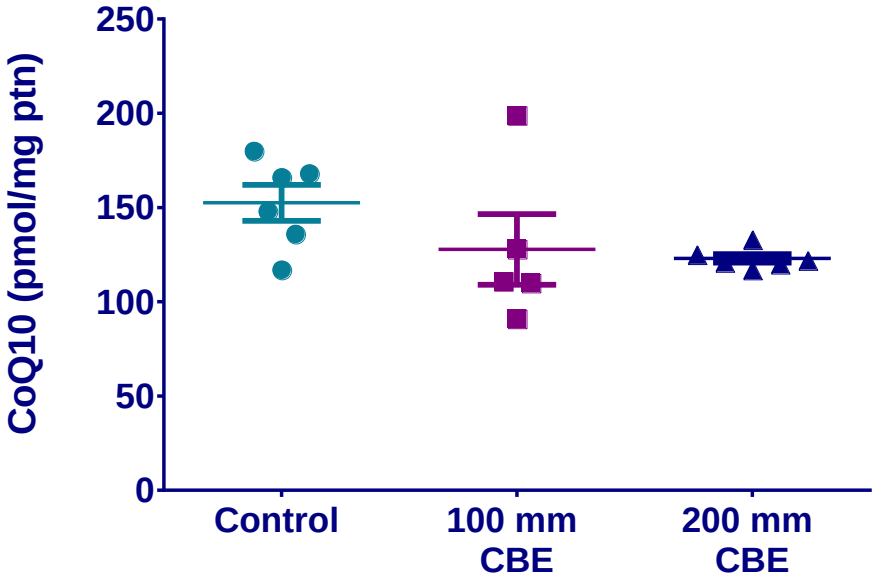

Figure 5.12 - Effects of GBA1 inhibition on SHSY5Y CoQ10 status.

The data is summarised in **Table 5.2** which demonstrates that GBA1 was over 99% inhibited in the cells cultured with CBE, while the control enzyme beta-galactosidase was unaffected.

Table 5.2 - Effects of GBA1 inhibition on SHSY5Y CoQ10 status.

No significant difference was observed between the three conditions (n=6).

	CBE (mM)		
	0	**100**	**200**
CoQ10 (pmol/mg protein)	152.5 +/- 9.5	127.8 +/- 18.7	123.0 +/- 2.3
GBA1 (nmol/hr/mg protein)	281.7 +/- 18.3	0.9 +/- 0.21	0.8 +/- 0.08
Beta-galactosidase (nmol/hr/mg protein)	609 +/- 55	587 +/- 108	513 +/- 87

5.5.5 Leucocyte CoQ10 status in GD and controls

CoQ10 levels were higher but not significantly in leucocytes from patients with Gaucher disease compared to controls (**Table 5.3**).

Table 5.3 - Leucocyte CoQ10 in GD and control plasma.

	Controls n=4	**GD n=4**	
CoQ10 (pmol/mg ptn)	68.1 ± 13.2	83.15 ± 5.09	P=0.329
BGAL (nmol/h/mg)	205 ± 49.3	286 ± 18.8	P=0.173
GBA1 (nmol/h/mg)	7.7 ± 1.6	0.70 ± 0.005	P=0.005
GBA2 (nmol/h/mg)	0.3 ± 0.2	0.6 ± 0.5	P=0.588

5.5.5.1 Citrate synthase

Citrate synthase activity was lower in the GBA1 inhibited cells compared to controls but the difference was not significant. There was a slight decrease in citrate synthase activity in the GBA1 inhibited cells with increasing levels of H_2O_2 but the activity at either 0.1 or 0.2 mM H_2O_2 was not significantly different to the control group (**Table 5.4**).

Table 5.4 - Effects of H_2O_2 on citrate synthase activity.

SH-SY5Y cells were treated with 0.1, 0.2, mM H_2O_2 for 24 hours after 14 days pre-treatment with CBE (n=3).

H_2O_2 (mM)	Control cells	GBA1 inhibited cells
0	89.6 +/- 5.1	85.3 +/- 10.8
0.1	91.3 +/- 2.9	77.4 +/- 16.3
0.2	84.7 +/- 5.4	76.5 +/- 13.5

5.6 Discussion

The principal aims of this chapter were to evaluate potential interactions between the lysosome, mitochondrion and oxidative stress, i.e. to identify potential new mechanisms pertinent to our understanding of the pathogenesis of PD.

Under the conditions employed, and using H_20_2 as a source of oxidative stress, exposure of the neuronal cells resulted in a dose dependent loss in cell viability. This is consistent with previous reports using MTT and H_20_2 [274]. Since the MTT assay is reported to reflect mitochondrial dehydrogenase activity because the tetrazolium ring is cleaved in active mitochondria [273], this finding is also consistent with observations that report, in a number of cell types, that mitochondrial enzymes are susceptible to oxidative damage [264, 265]. Repeating the dose response curve on a background of inhibition of GBA1, GBA2 or GBA1 plus GBA2 did not alter the sensitivity of the cells to oxidative stress. The results presented here could suggest that the loss of GBA1 associated in PD may not exacerbate any oxidative stress mediated neuronal damage. Similarly, under the conditions employed, GBA2 does not appear to contribute to the process.

Oxidative stress has also been suggested be a mechanism for the loss of mitochondrial respiratory chain enzyme activities in PD, i.e. these enzymes are prone to direct oxidative damage as well as indirect effects due to oxidation of cardiolipin, a phospholipid situated on the inner mitochondrial membrane [275]. In contrast to this group of enzymes, GBA1 activity was not impaired following exposure to H_20_2. This result implies that loss the loss of GBA1 activity reported in PD is not arising as a consequence of oxidative stress. Similarly none of the other lysosomal enzymes studied displayed loss of activity following H_20_2 exposure. Moreover, some activities significantly increased.

As discussed in Chapter 4, GBA2 activity could play a role in dictating cellular vulnerability and may be a factor to consider with regards to why some but not all individuals with GBA1 mutations develop PD. Whilst inhibition of GBA2 did not influence cell viability, loss of activity resulted in a dose response increase in GBA1 activity following H_20_2 exposure. Whilst the mechanism for this effect is not known,

these data provide further evidence for an interaction between these two enzymes and implies that an up-regulation of sphingolipid metabolism occurs in response to oxidative stress, i.e. when GBA2 activity is diminished.

Total beta-hexosaminidase also showed a significant increase in activity following H_20_2 exposure. In contrast to GBA1 this increase occurred in the absence of any blockade of GBA2 activity. Total beta-hexosaminidase is also involved in sphingolipid metabolism and so these data may again point to an up-regulation in this pathway in response to oxidant exposure. Total beta-hexosaminidase has been found to be significantly increased in CSF from PD patients [276] and also in plasma from most but not all GD patients [40]. However, others have found no increase in plasma levels [245]. Analysis here of plasma from three GD patients found that 2 had elevated levels while the other was in the normal range and similar to simultaneously assayed unaffected control samples. It is of interest to note that total beta-hexosaminidase is involved in the production of GM3 ganglioside, which plays a protective role in neurodegeneration processes taking place in PD [276]. Total beta-hexosaminidase has also been found to be higher in fibroblasts from GD-PD patients but not in iPD patients [277]. The report of elevated beta-hexosaminidase activity in CSF from PD patients has led to the suggestion that assessment of activity could be a useful biomarker for PD [278]. The mechanism for the elevation in enzyme activity or whether the elevation is harmful or beneficial is not known. However, in view of the reported involvement of oxidative stress in PD and the data presented here, it is possible that this enzyme may be reflecting increased oxidant exposure. Consequently, it would be of interest to evaluate total beta-hexosaminidase activity in more detail in other models of oxidative stress and in conditions where it is implicated, e.g. Alzheimer's disease, multiple sclerosis and inborn errors of glutathione metabolism. The findings of elevated plasma total beta-hexosaminidase in plasma from GD patients demonstrated here may be secondary to lysosomal dysfunction but requires further work

In contrast to the above enzymes, beta galactosidase was unaffected by any of the inhibitor treatments, despite being involved in sphingolipid metabolism. This result therefore suggests that the observed effects are not general to all lysosomal

enzymes. However, the response to H_20_2 is not specific to the sphingolipid pathway as alpha glucosidase activity was also affected. The effect was comparable to that for GBA1. It is of note that both enzymes are glucosidases and that their activities were particularly increased, following H_20_2 exposure and when GBA2 activity was impaired. Alpha glucosidase is involved in lysosomal glycogen catabolism and hence liberation of glucose [4, 279]. Although present in much higher amounts in glial cells, it has been demonstrated that neurons have an active glycogen metabolism that protects cultured neurons from hypoxia-induced stress [280]. Increased oxidative stress, as studied here, may result in an increased need for glycogen derived glucose. The interaction between GBA2 and this glucosidase also requires further investigation.

Importantly, when studying the effects of CBE and NBDNJ, alpha glucosidase activity was found to be inhibited. This is perhaps not surprising as both inhibitors target glucosidases [281-283]. However, it is important in view of the results reported here, that the relatively non-specific nature of these two inhibitors are considered, particularly as, in the case of CBE, they are used to model disease conditions [228, 284-286].

Loss of mitochondrial respiratory chain enzyme activities, particularly at the level of complex I, is well reported in PD. The consequences of this will include impaired energy metabolism and oxidative stress [287]. Rotenone is an established complex I inhibitor and was used here under conditions previously documented by our laboratory to inhibit complex I and increase reactive oxygen species generation [288]. Using this protocol, treatment of the neuronal cells did not result in any loss of GBA1 activity. These data may suggest that loss of complex I does not contribute to the impaired GBA1 activity reported in idiopathic PD.

To further evaluate any potential interactions between lysosomal and mitochondrial function, the effects of GBA1 inhibition on parameters related to mitochondrial metabolism and content were studied. CoQ10 levels were unaffected by the treatment suggesting that diminished GBA1 activity does not contribute to loss of CoQ10 availability reported in PD. Similarly, assessment of

mitochondrial content, as judged by citrate synthase activity, was not altered by the CBE treatment. For these parameters at least there appears to be no interaction between the lysosome and mitochondrion.

5.7 Conclusions

Oxidative stress is a cause of loss neuronal viability, a situation that does not appear to be affected by GBA1 or GBA2 activity. Furthermore, oxidative stress itself does not have any discernible negative effect on GBA1 activity and so does not provide an explanation for the loss of GBA1 activity associated in idiopathic PD. Lysosomal glucosidases however increase their activity in response to oxidative stress and this is particularly marked when GBA2 is inhibited. An interaction between glucosidases in response to cellular stress is therefore indicated and warrants further evaluation. Beta hexosaminidase also increased in response to oxidative stress and again may point to a stress response. Oxidative stress may provide a mechanism for the increased activity of this enzyme reported in the CSF of patients with PD. With regards to the effect of impaired GBA1 activity on mitochondrial status, no effect upon CoQ10 and mitochondrial enrichment was observed. Further work is still required to ascertain the mechanism for the loss of GBA1 activity and protein reported in idiopathic PD.

Chapter 6 Western blots of GBA1 and GBA2

6.1 Introduction

The interplay between GBA1 and GBA2 has so far in this thesis, been evaluated by assessment of enzyme activity. In Chapter 4, decreased brain GBA1 activity, in a mouse model of GD, was reported to be associated with an increase in GBA2 activity. Furthermore, for patients with GD, a proportion of individuals were found to display increased leucocyte GBA2 activity. These data may therefore imply an interaction between these two enzymes and raises the possibility that when activity of one of one of these enzymes is compromised, the cellular response is to divert substrate to the other.

In order to explore this possibility further at the protein level, western blotting was developed and reported in this chapter for GBA1 and GBA2. Initial data are also reported with regards to the effects of pharmacological inhibition, with CBE, on expression of GBA1 and GBA2.

The western blotting method was based on published methods for GBA1 [192] and GBA2 [228] and departmental experience of western blotting for the alpha-glucosidase enzyme in Pompe disease. Optimisation for the thesis included checking the protein loading required, the dilution of antibodies required and the time of exposure required for visualisation of the various bands.

6.2 Methods

Human fibroblasts (controls and patients with GD) or SH-SY5Y cells were cultured as described in section 2.12. SH-SY5Y cells had been exposed to 100 μM of CBE for 11 days prior to harvest. Harvested fibroblasts or SH-SY5Y cells were washed three times in PBS. After the last wash all PBS was removed and 50 μL of inhibitor solution (5 μL Halt Protease Inhibitor (PI) Cocktail and 5 μL EDTA added to 0.5 mL DD H_20) was added. If not being assayed immediately the sample was snap frozen until required. When required, the sample was thawed and the cells sonicated at

amplitude 6 for 10 seconds. The protein levels were determined using the BCA assay described in section 2.11, and the samples were then diluted down to 2.0 μg/ μL in inhibitor solution. The samples were prepared for assay adding 4X LDS (lithium dodecyl sulphate) equivalent to 25 % of the final volume and 10X DTT (dithiothreitol) equivalent to 10 % of the final volume. The samples were then heated for 10 minutes at 70 ^{o} C.

Samples (25μL) were separated under denaturing conditions on a 4-12% Bis-Tris Gel in MOPS (3-(N-morpholino)propanesulfonic acid) running buffer at a constant voltage of 120V on ice for approximately 2 hours. Protein was then transferred to a PVDF membrane by electro transfer using the iBlot™ Dry Blotting System. After transfer the gel was rehydrated in water before staining in SimplyBlue SafeStain to confirm that the bands had run straight. The membrane was cut into three pieces as shown in **Figure 6.1** using the molecular marker as a guide so that the top piece contained GBA2, the middle piece GBA1 and the bottom piece GAPDH, before incubation of the three pieces of membrane in blocking buffer (5% non-fat dry milk in TBST (Tris-buffered saline tween-20)) for 1 hour.

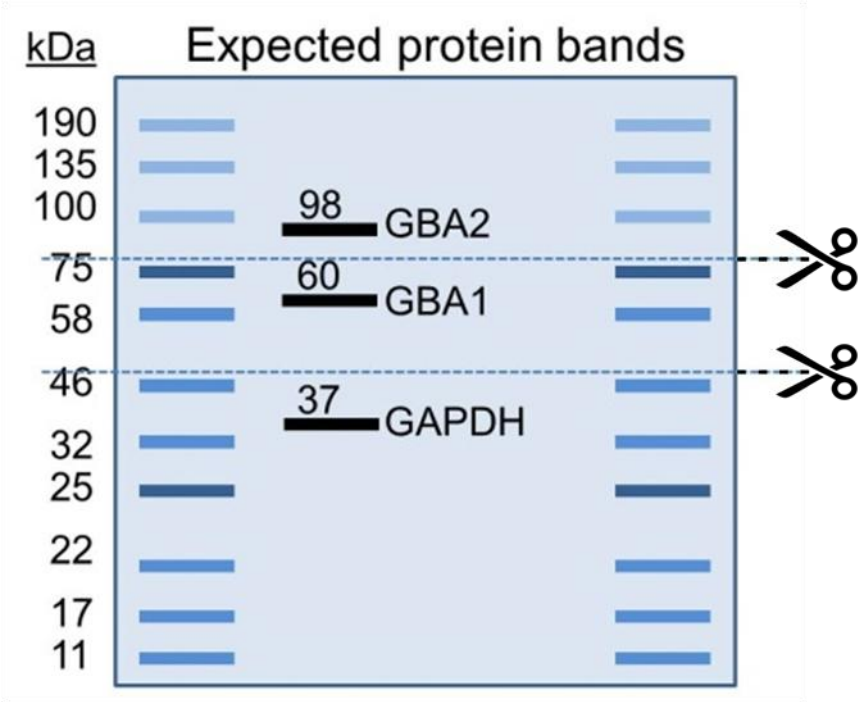

Figure 6.1 –Diagram showing the positions of the bands of interest on the membrane.

Positions based on their reported molecular weights i.e. GBA2 - Predicted band size : 98-104 kDa [289], GBA1- Predicted band size : 60 kDa [290] and GAPDH - Predicted band size : 37 kDa [291] and where the membrane was cut prior to blocking and probing.

Cutting the membrane allowed for simultaneous blocking and probing of the different proteins and produced cleaner final images.

Following blocking the appropriate piece of membrane was probed with the respective antibody overnight at 4 ^{o} C. All antibodies were diluted in blocking buffer. The top part of the membrane was probed with anti-GBA2 at 1:500 dilution, the middle part of the membrane with anti-GBA1 at 1:500 and the bottom part of the membrane with anti-GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) at 1:1200. GAPDH was used as the reference protein band to check that samples were evenly loaded, and to confirm the transfer of proteins at the blotting stage. The membranes were then washed 4 x 15 minutes in TBST before incubation with the secondary antibody; goat anti-mouse IgG-HRP (horseradish peroxidase) at 1:2000 for GBA1 mouse monoclonal antibody and donkey anti-rabbit IgG-HRP at 1:4000 for the GBA2 and GAPDH polyclonal antibodies. The membranes were then washed 4 x

15 minutes in TBST before visualisation of the bands. The membranes were incubated with ECL (Enhanced chemiluminescence) substrate according to the manufacturer's instructions and visualised by exposure to x-ray film. GAPDH was visualised in 3-5 minutes, GBA1 in 30 and GBA2 required 60 minutes.

6.3 Results

As an initial control experiment, Western blotting was performed on control and GD fibroblasts, i.e. to provide further validation of the assay with regards to identifying changes in GBA protein expression. For all control samples a clear GBA1 band was identified running at approximately 60 kDa. For the samples derived from GD patients, a faint band was only apparent for 1 individual (GD1). For the remaining two patients no GBA1 band could be observed. The molecular marker (Blue Pre-stained Protein Standard, Broad Range (11-190 kDa) ladder) is not visible on the membrane.

Assessment of enzyme activity revealed very low results for the GD samples (results displayed in **Figure 6.2**. It is noted that the patient with the highest residual enzyme activity was the sample in which a faint GBA1 protein band could be observed.

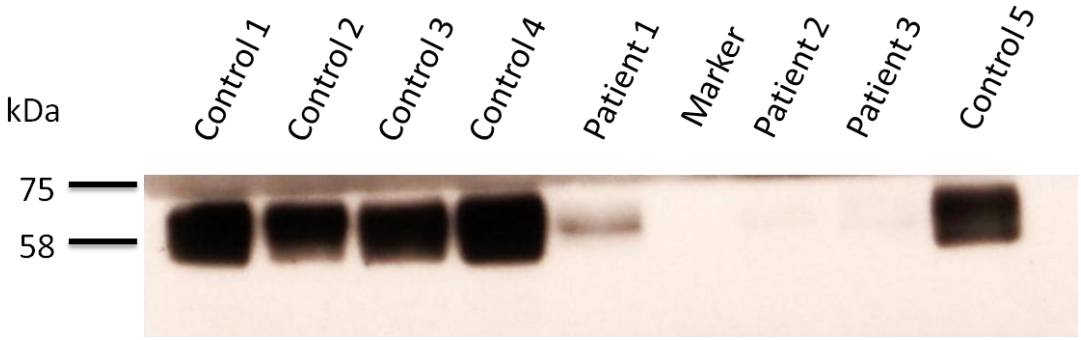

Figure 6.2 - Western blot of fibroblast GBA1.

Table 6.1 - GBA1 and beta-galactosidase (B-GAL) activities of fibroblast samples shown in western blot (Figure 6.2).

Sample	GBA1 (nmol/mg/ptn)	B-GAL (nmol/mg/ptn)
Control 1	857	1720
Control 2	710	1313
Control 3	853	1596
Control 4	1077	2560
Patient 1	157	1878
Patient 2	42	1696
Patient 3	44	1437
Control 5	520	1061

Concerning SH-SY5Y cells, GBA1, GBA2 and GAPDH protein bands could be identified following Western blotting (**Figure 6.3**). Inhibition of GBA1, by CBE 11 days, had no apparent effect on the expression of GBA2 (**Figure 6.3**). However, under such conditions, expression of GBA1 appeared to increase (**Figure 6.3**).

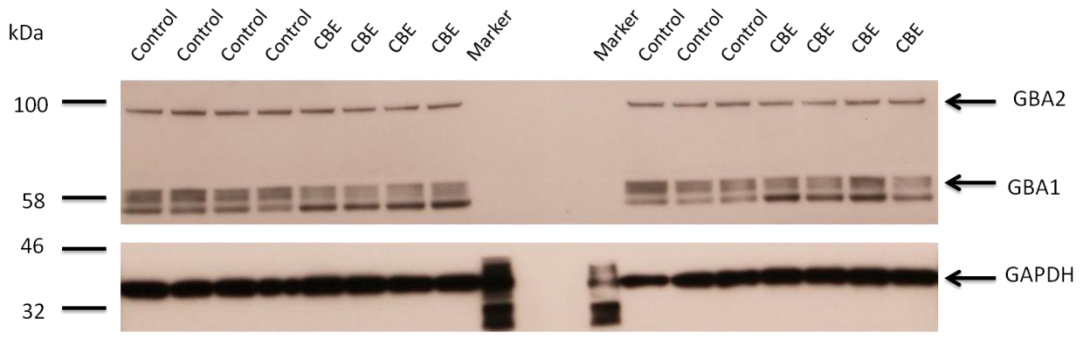

Figure 6.3 - Western blot of SH-SY5Y GBA2, GBA1 and GAPDH.

The effectiveness of CBE to create GBA1 deficiency was confirmed with the findings of significantly decreased GBA1 activity in the inhibited cells compared to control cells, while the control enzyme, beta-galactosidase was comparable between the two groups (Table 6.2).

Table 6.2- GBA1 and beta-galactosidase (B-GAL) activities of SH-SY5Y samples shown in western blot (Figure 6.3).

Sample	GBA1 (nmol/mg/ptn)	B-GAL (nmol/mg/ptn)
Controls (n=7)	106.1 ± 4.8	381± 18
CBE inhibited cells (n=8)	0.6 ± 0.2	390 ± 22
Significance	<0.0001	NS

6.4 Discussion

Conditions have been described here to permit western blotting for GBA1 and GBA2 in both human cultured fibroblasts and the neuronal, SH-SY5Y cell line. The procedure described here was able to demonstrate absence of a GBA1 band in two patients with GD and an apparently very faint band in another GD patient. Such results give a degree of validation of the assay. Whilst the patient with the faint band had the highest residual enzyme activity, further studies will be required to ascertain the relationship between individual patient genotype and protein expression. With further refinement to include quantification (densitometry and relationship to GAPDH loading), western blotting may also have a role in the diagnostic laboratory e.g. when genetic variants of unknown significance are reported or recording the potential efficacy of novel treatment strategies such as chaperone type molecules.

Concerning the SH-SY5Y cells, the presence of GBA1 and GBA2 bands could clearly been seen. It was noted that two bands were apparent with regards to GBA1 staining. Whilst the reason for this is not yet clear, this phenomenon has been reported before in brain and SH-SY5Y cells [192].

Exposure of SH-SY5Y cells to CBE resulted, as expected, in a marked loss of GBA1 activity. Inspection of the western blots, after such treatment did not appear to result in any marked difference in the intensity of GBA2 staining. Visualising GAPDH did not point to any marked discrepancies between control and CBE treated cells. These preliminary data, therefore do not point to any marked up-regulation in GBA2 as a consequence of the block of GBA1 activity. This result is consistent with the lack of effect upon GBA2 enzymatic activity reported in section 4.4.7.

In contrast to GBA2, CBE treatment appeared to increase the intensity of the GBA1 band in all treated cells, i.e. suggesting GBA1 up-regulation when activity is inhibited by CBE for 11 days. CBE is a very widely used and accepted inhibitor to model GD in cellular and animal models [228, 284-286]. The cellular consequences of this apparent up-regulation, if confirmed, should be considered in such models. Concerning the mechanism for this observation, it remains to be demonstrated

whether this effect is in response to substrate (GlcCer) accumulation or a cellular deficiency of the GBA1 product, ceramide.

Chapter 7 Analysis of ceramides by UPLC-MS/MS to examine the effects of GBA1 and/or GBA2 inhibition.

7.1 Introduction

7.1.1 Glucosylceramide and ceramide

Glucosylceramide (GlcCer) was first identified as the accumulating lipid in GD in 1934 and is now known to accumulate in every tissue where its levels have been measured [26]. GlcCer is deacylated by lysosomal acid ceramidase to form glucosylsphingosine (GlcSph) [225]. GlcSph is usually not detectable in tissue from unaffected individuals, but significantly accumulates in GD but to lower levels than GlcCer. The levels of GlcSph have been found to be higher in the brains of type 2 and 3 patients with GD suggesting a potential pathological role for this lipid in types 2 and 3 GD [26]. GlcSph is broken down into glucose and sphingosine in the lysosome by GBA1 and by GBA2 outside the lysosome [26, 225]. In unaffected individuals, GlcCer is broken down into glucose and ceramide in the lysosome by GBA1 and by GBA2 outside the lysosome [110, 178]. The simplest sphingolipid is ceramide which consists of a fatty acid residue attached to a sphingosine backbone at C-2 by an amide link [12]. Ceramide functions as a precursor for more complex glycosphingolipids such as sphingomyelin or cerebrosides such as GlcCer [13]. Ceramide and sphingosine are catalyzed to ceramide-1-phosphate and sphingosine-1-phosphate respectively by their specific kinases [225]. See **Figure 7.1** for a summary of ceramide metabolism. Ceramide can induce apoptosis, but its conversion to sphingosine 1-phosphate or glycosphingolipids induce cell proliferation [292]. The control of cell fate by these two inter-convertible lipids has been called the sphingolipid rheostat or sphingolipid biostat [293]. Ceramide can also induce necroptosis [294] which is a feature of both GD and Krabbe leucodystrophy [19].

Ceramide has multiple isoforms with variable acyl chain length comprised of 14 to 26 carbons found in most cells [295] although acyl chain length of up to 36 carbons form a major portion of ceramide compounds in the epidermis [296]. There are six

different human ceramide synthases identified with different specificities, producing ceramides/dihydroceramides with differing chain lengths [295]. However the salvage pathway, whereby GBA1 produces ceramide from GlcCer is the most energy efficient method of synthesising ceramide in post-mitotic cells [27].

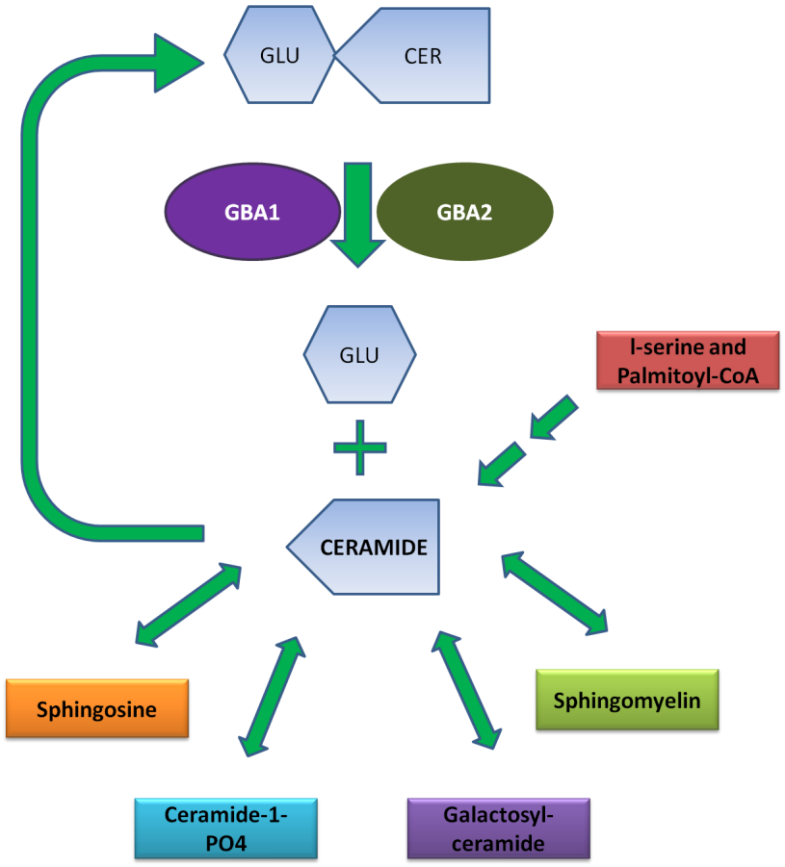

Figure 7.1 Summary of ceramide metabolism.

Ceramide is the centrepiece of the sphingolipid metabolic pathway and can be produced through hydrolysis of GlcCer by GBA1 and GBA2, through hydrolysis of galactosylceramide by galactocerebrosidase and through hydrolysis of sphingomyelin by sphingomyelinase. Ceramide can also be synthesized from ceramide-1-phosphate through the action of ceramide-1-phosphate phosphatase. Finally, ceramide can be catabolised to sphingosine and further to sphingosine-1-phosphate. Ceramide can also be synthesized de novo from l-serine and palmitoyl-CoA (adapted from [297] and [298].

Different isoforms of ceramide have different functions and are present in different amounts throughout the body. C22:0 ceramide is the major isoforms in hepatocytes, lungs and intestine [299]. C18:0 and C24:0 ceramide are the dominant species in the central nervous system. C18:0 ceramide is vital for the normal development of neurons while C24:0 ceramide is crucial for myelin production in oligodendrocytes [295].

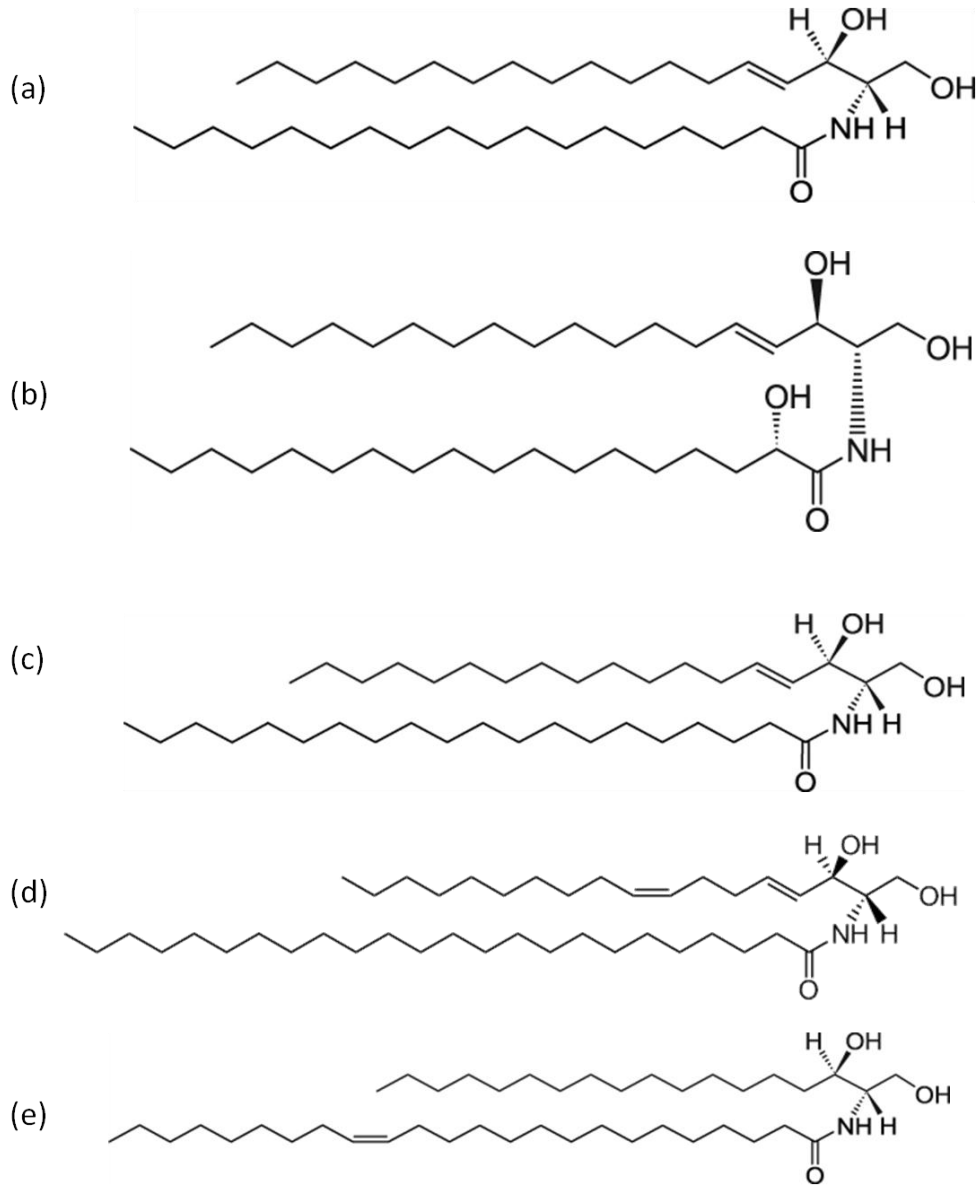

Figure 7.2 Examples of ceramides:

(a) C18:0 ceramide; (b) C18:0-OH ceramide; (c) C20:0 ceramide: (d) C24:0 ceramide; (e) C24:1 ceramide [300].

There is evidence that ceramide plays a role in both GD and Lewy body diseases. Regarding ceramide metabolism in GD, plasma levels have been determined with reports of no changes compared to matched controls [301], or increases that decline after ERT [302]. In a study of non-GBA1 carrying PD patients, plasma ceramide levels were reported to be higher in patients compared to controls with the highest levels in those with cognitive impairment compared to those without [303]. In sporadic Parkinson's disease, GBA1 activity and protein levels are reduced in brain areas that accumulate α-synuclein and this appears to be associated with a reduction in ceramide levels [270]. In a *LRRK2* mouse model, brain ceramide levels are significantly higher in knockouts (*Lrrk2*$^{-/-}$) compared to wildtype (*Lrrk2*$^{+/+}$) mice, suggesting that the absence of *LRRK2* has an impact on ceramide metabolism. While the total ceramide levels are elevated in the knock-outs, the intra-class ceramide distribution i.e. the relative amounts of different acyl chains was not different compared to wildtype [304]. Genes involved in Neurodegeneration with Brain Iron Accumulation (NBIA), have roles in ceramide metabolism. The pantothenate kinase gene (PANK2) is a regulatory enzyme for co-enzyme A biosynthesis which is essential for the *de novo* pathway for ceramide production. While, PLA2G6 (phospholipase A2 group VI) is a phospholipase that promotes ceramide generation via hydrolysis of sphingomyelin [297]. It is not only the overall amount of ceramide that is important but also the relative amounts of the different ceramide species as defined by their fatty acyl chain length [305]. It has been shown that the middle frontal gyrus in patients with Alzheimer's disease (AD), a brain region with extensive β-amyloid plaques and tangles, accumulates more C24:0 ceramide in addition to cholesterol relative to age-matched control samples [295]. Elevated ceramide levels have been shown to significantly enhance the level of free radicals and decrease the viability of SH-SY5Y cells [306]. Metabolism of ceramide and GlcCer been proposed to play an important role in the control of energy homeostasis [232].

It is uncertain if GlcCer mediates it pathological effects in GD entirely from within the lysosome or whether some can escape and mediate effects in the cytosol or with other organelles [26]. If excess GlcCer does egress the lysosome, its metabolism by GBA2 would release ceramide which may have deleterious effects.

Thus, extra-lysosomal metabolism, via GBA2, could lead to maintenance or elevation of total cellular ceramide content in a GBA1 deficiency. In contrast, loss of GBA2 activity could lead to even higher GlcCer levels but lower or normal ceramide levels.

7.1.2 Ultra Performance Liquid Chromatography (UP)LC-MS/MS

Mass spectrometers (MS) function by converting the analyte molecules in the sample of interest to a charged or ionised state, followed by the analysis of the ions and any fragment ions that are produced during the ionisation process, based on their mass to charge ratio (m/z). Ultra Performance Liquid Chromatography (UP)LC-MS/MS is a technique which combines the physical separation abilities of liquid chromatography often used in combination with the mass analysis power of mass spectrometry. This technique possesses particularly high sensitivity and specificity for quantitation of analytes in complex mixtures [307].

There are many types of mass analysers, but the work described in this thesis was conducted using a triple quadrupole mass spectrometer. A quadrupole analyser consists of a set of four parallel metal rods. Using a combination of alternating and varying radio frequency (RF)/direct current (DC) voltages allows the transmission of a narrow band of m/z values along the axis of the rods. By varying the DC voltages with time it is possible to scan across a range of m/z values, resulting in a mass spectrum. A triple quadrupole mass spectrometer is a tandem MS method which consists of three quadrupoles. The first and third quadrupoles act as mass filters which separate ions based on their m/z ratios. The second quadrupole causes analyte fragmentation via interaction with a collision gas. Altering the voltage of the first quadrupole enables the process to be manipulated in a way such that only ions of particular m/z can traverse the quadrupole. The method can be used to obtain structural information or for quantitation of analytes. Triple-quadrupole mass spectrometry techniques coupled with UPLC, offers improved selectivity, improved signal-to-noise ratio, lower quantitation limits and increased accuracy over other methods [307, 308].

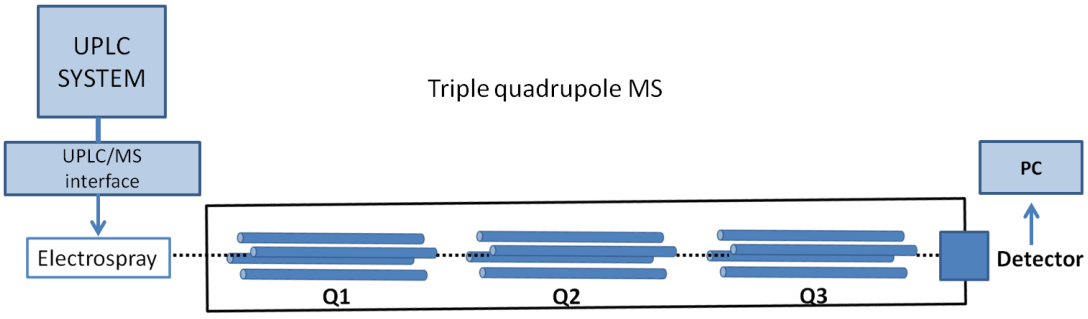

Figure 7.3 - Schematic diagram of a Ultra Performance Liquid Chromatography (UP)LC-MS/MS system

Internal standard is added to the samples prior to UPLC-MS/MS analysis. An internal standard chemically mimics a metabolite of interest, having similar chemical structure but usually containing heavier isotopes such as deuterium (^{2}H) or ^{13}C labels. The stable isotope internal standards have the same extraction recovery, ionization response and the same chromatographic retention time as the compound to be quantified but can only be distinguished from the endogenous compound of interest due to the difference in their masses. Using this principle the mass spectrometer was used to quantify different ceramide isoforms in what is termed multiple reaction monitoring (MRM). MRM provides increased specificity by monitoring a parent and one or more of its product ions simultaneously after fragmentation. The concentration of the analytes are determined through the use of calibrator curves and relating the analyte/IS peak ratios [307, 308].

7.1.3 AIMS

In view of the potential role of perturbation of ceramide metabolism in GD, and possibly PD, the aim of this chapter was to begin to document the effects of inhibition of neuronal (SH-SY5Y) GBA1, GBA2, or both, on ceramide species. In order to achieve this, a targeted UPLC-MS/MS method was developed to quantitative different ceramides.

7.2 Experimental protocol

SH-SY5Y cells were cultured as described in section 2.12.1 but with the addition of 100 μM CBE, 10 μM NBDNJ or both 100 μM CBE and 10 μM NBDNJ added to the medium. Control cells had no inhibitor added to the medium. Cells were harvested and washed as described in section 2.12.1 after 17 days exposure to the inhibitors.

7.2.1 Sample preparation

Cell pellets were disrupted by sonication at amplitude 6 for 10 seconds and protein levels were determined using the BCA method as described in section 2.11. Samples were then diluted in deionised water where necessary so that all were normalised to the same protein concentration. Lipids were extracted from the samples using the method of Bligh and Dyer [309]. Extraction solvent was prepared by adding internal standard (N-Octadecanoyl-D_3-D-erythro-sphingosine) to a 1:2 (v/v) chloroform/methanol mixture. For each sample, 0.75 mL of extraction solvent was added to 0.2 mL of sample in an Eppendorf tube and vortexed vigorously. Then 0.25 mL of chloroform was added and vortexed vigorously before the addition of a further aliquot of 0.25 mL of deionised water and a final vortex. Samples were then centrifuged at 3000g for 5 minutes at room temperature which resulted in a biphasic solution of an upper methanolic phase and a lower organic phase. The lower organic phase (chloroform) containing the ceramides was recovered into a glass autosampler vial and evaporated to dryness under nitrogen. The samples were reconstituted in 50μl of methanol, and the vials capped, prior to analysis by MS.

7.2.2 UPLC-MS/MS analysis

Mass spectrometry was carried out in the UCL Great Ormond Street Institute of Child Health. Previous work had been performed to test the extraction method and identify the required parameters required for the analysis of ceramides.

UPLC-MS/MS analysis was performed on a Waters Xevo^{TM} TQ-S MS system operated in negative ion mode coupled to a Waters ACQUITY UPLC (Manchester, UK). The column temperature was kept at 40 °C.

A 10 µL injection of sample was passed through a using an ACQUITY UPLC BEH C8 VanGuard pre column (130Å, 1.7 µm, 2.1 mm X 5 mm) and C8 column (130Å, 1.7 µm, 2.1 mm X 50 mm) using mobile phase (A) 0.01% formic acid in ddH_2O and (B) 0.01% formic acid in methanol. Initial conditions were 90% A and 10% B for 5 minutes. At 5.01 minutes mobile phase B was increased to 100% over 1 minute and maintained at 100% for a further 2 minutes followed by a return to initial conditions (90% A, 10% B) for the final 3 minutes of the eleven minute method. An injection of 10 µL of blank methanol was run after every two samples to ensure minimised carryover between samples.

The transitions or MRMs used for identifying the peaks including the internal standard (C18:0-D3 ceramide (N-Octadecanoyl-D3-D-erythro-sphingosine)), are listed in **Table 7.1**.

Table 7.1 List of the multiple reaction monitoring (MRM) transitions used.

Isoforms	Formula	Parent ion m/z	Daughter ion m/z
C16:0-OH ceramide	$C_{34}H_{66}NO_4$	552.4997	296.2997
C18:0 ceramide	$C_{36}H_{71}NO_3$	564.6391	308.4265
C18:0-D3 ceramide	$C_{36}H_{69}D_3NO_6$	567.5519	311.2905
C18:0-OH ceramide	$C_{36}H_{71}NO_4$	580.5702	253.3707
C20:1 ceramide	$C_{38}H_{73}NO_3$	590.5518	334.3518
C20:0 ceramide	$C_{38}H_{75}NO_3$	592.5674	336.3674
C20:0-OH ceramide	$C_{38}H_{75}NO_4$	608.5623	281.3623
C24:1 ceramide	$C_{42}H_{81}NO_3$	646.6536	390.4832
C24:0 ceramide	$C_{42}H_{83}NO_3$	648.733	349.4593

Automated UPLC profiles and MRM transitions were created and samples analysed using an auto sampler. Data was acquired from the UPLC/MS-MS using Masslynx™ 4.1 Software (Waters). Automated analysis was performed using Integrated

chromatogram traces using TargetLynx™ software as shown in **Figure 7.4**. These were provided with peak information, such as response and signal-to-noise (S/N) ratio. Quality control was maintained by random selection of various chromatograms and manual processing. This included checking the peak area in chromatograms and manually adjusting the area under a peak to fit the area under a peak if needed.

7.3 RESULTS

Using the conditions described above it was possible to identify ceramide species in the SH-SY5Y cell preparations. An example of a chromatogram is displayed in **Figure 7.4** and graphs of the results are displayed In **Figure 7.5** and **Figure 7.6**. The actual amount of any specific ceramide was not quantified. The results presented are the peak area of the analyte of interest in a sample divided by the peak area of the internal standard for that sample. This allowed for comparison of the relative amounts of ceramides between different samples in the four conditions. Only the eight ceramide isoforms with a signal to noise ratio of 3:1 were included in the analysis.

SH-SY5Y cells had been cultured with no inhibitors (controls), with 100 μM CBE to inhibit GBA1, with 10μ μM NBDNJ to inhibit GBA2 and with both 100 μM CBE and 10 μM NBDNJ to inhibit both GBA1 and GBA2.

C24:1 ceramide was the predominant isoform detected in all cells with C24:0 ceramide the next most abundant and all other isoforms detected were present at much lower relative levels. Concerning the levels of ceramide and its isoforms following inhibition of GBA1, GBA2 or both, no significant difference in levels was apparent. However, apart from C20:0-OH ceramide, there was a trend for all isoforms to be higher in the inhibited cells **(Table 7.2)**.

Table 7.2 - Summary of ceramide results in SH-SY5Y cells.

Control = uninhibited cells, CBE = GBA1 inhibited cells, NBDNJ = GBA2 inhibited cells and CBE & NBDNJ = GBA1 and GBA2 inhibited cells. Values displayed are the peak area of the ceramide of interest divided by the peak area of the internal standard for that sample.

Ceramide	Controls	CBE	NBDNJ	CBE & NBDNJ
C16:0-OH	0.37 ± 0.06	0.47 ± 0.03	0.53 ± 0.04	0.50 ± 0.10
C18:0	0.44 ± 0.02	0.51 ± 0.03	0.58 ± 0.02	0.70 ± 0.12
C18:0-OH	0.55 ± 0.04	0.66 ± 0.01	0.63 ± 0.05	0.75 ± 0.13
C20:0	0.34 ± 0.03	0.37 ± 0.02	0.40 ± 0.01	0.48 ± 0.06
C20:0-OH	0.89 ± 0.06	0.87 ± 0.08	0.70 ± 0.01	0.71 ± 0.17
C20:1	0.006 ± 0.001	0.009 ± 0.002	0.012 ± 0.001	0.013 ± 0.003
C24:0	46.7 ± 1.4	54.7 ± 2.2	55.8 ± 3.1	65.2 ± 9.1
C24:1	82.2 ± 6.2	109.1 ± 2.7	109.1 ± 6.7	119.7 ± 16.9
Total	131.5 ± 7.0	166.7 ± 3.9	167.8 ± 9.7	187.8 ± 26.5

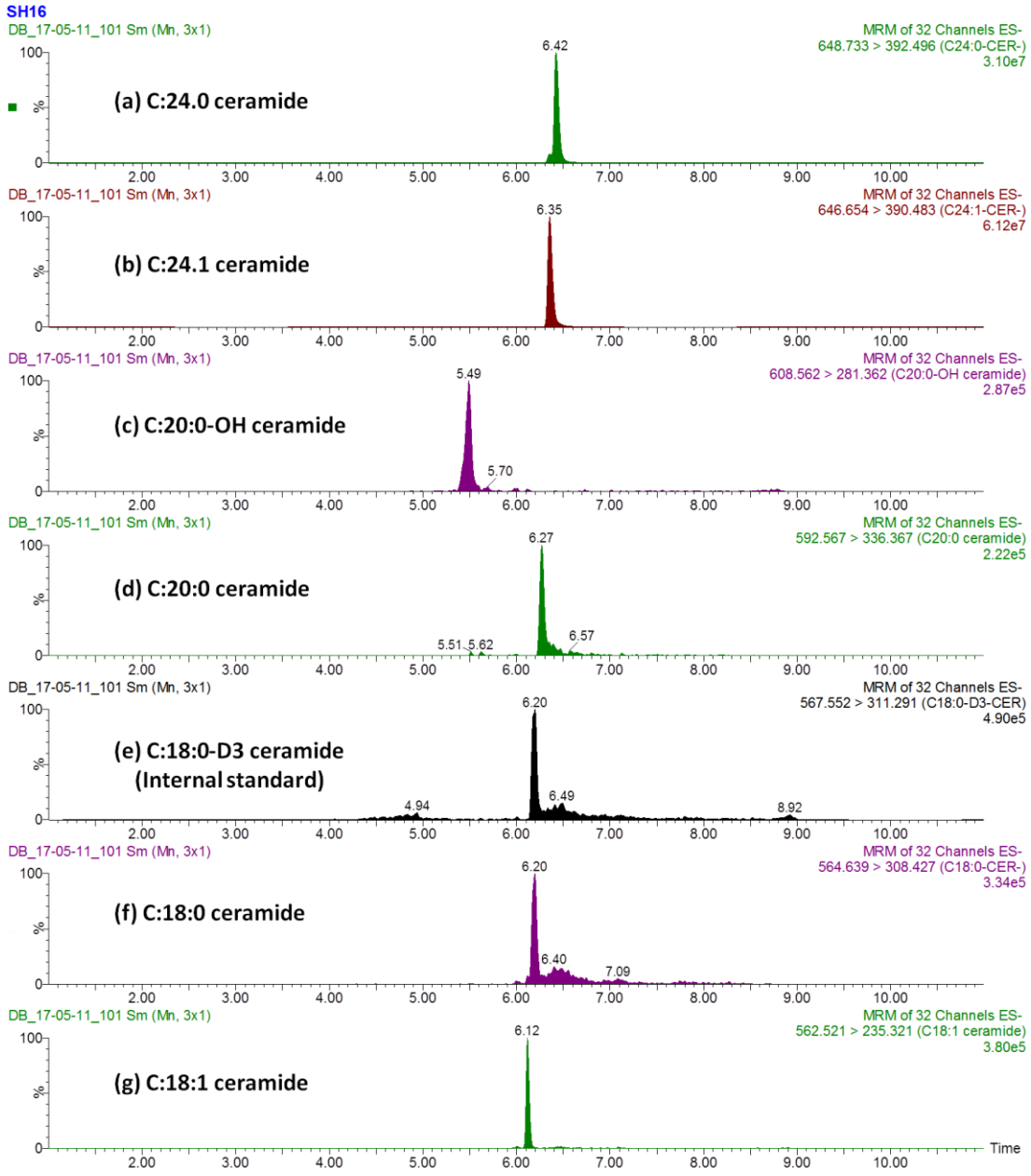

Figure 7.4 - Example of a typical chromatogram of various ceramide species detected in the SH-SY5Y cells.

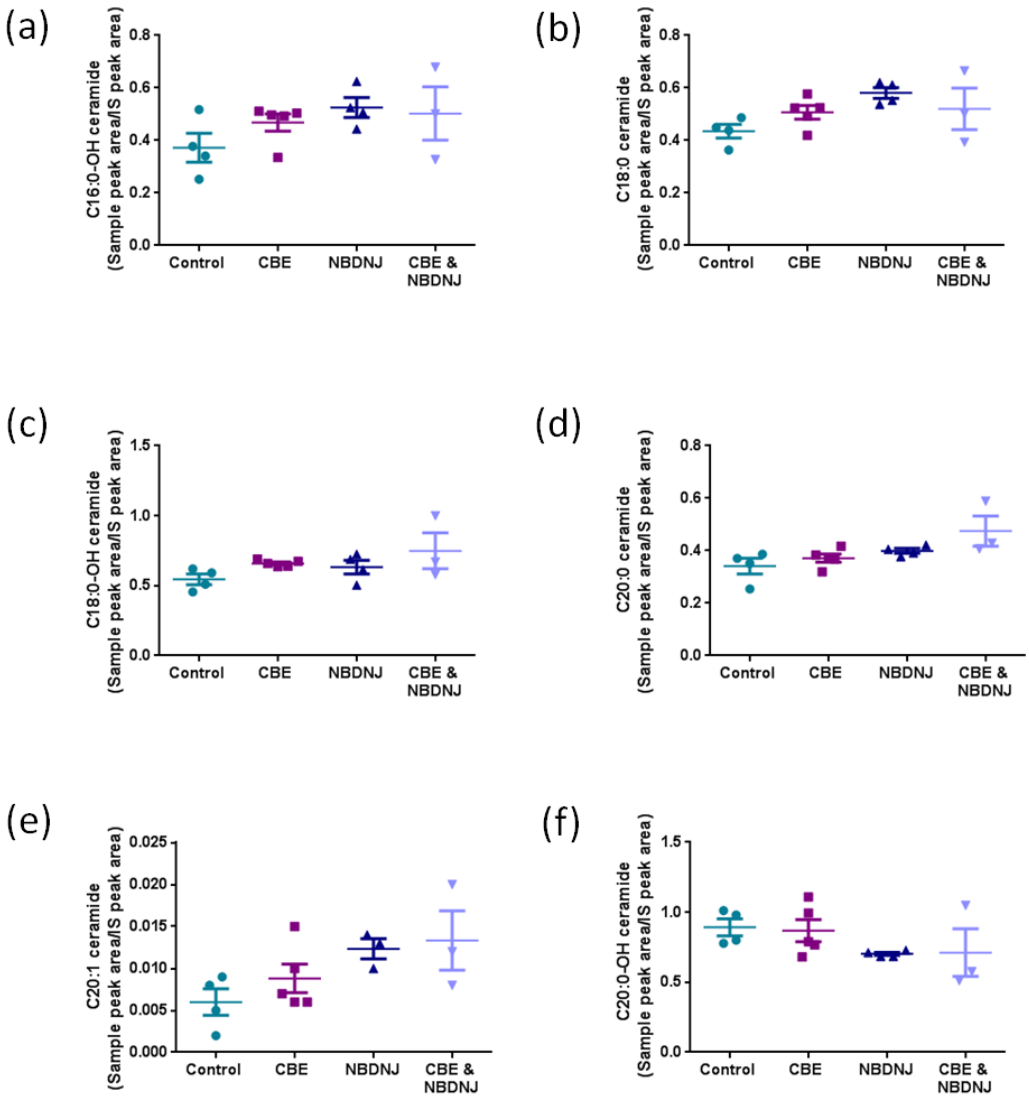

Figure 7.5 - Results of (a) C16:0-OH, (b) C18:0, (c) C18:0-OH, (d) C20:0, (e) C20:1 and (f) C20:0-OH ceramide analysis in SH-SY5Y cells.

Control = uninhibited cells, CBE = GBA1 inhibited cells, NBDNJ = GBA2 inhibited cells and CBE & NBDNJ = GBA1 and GBA2 inhibited cells. Values displayed are the peak area of the ceramide of interest divided by the peak area of the internal standard for that sample.

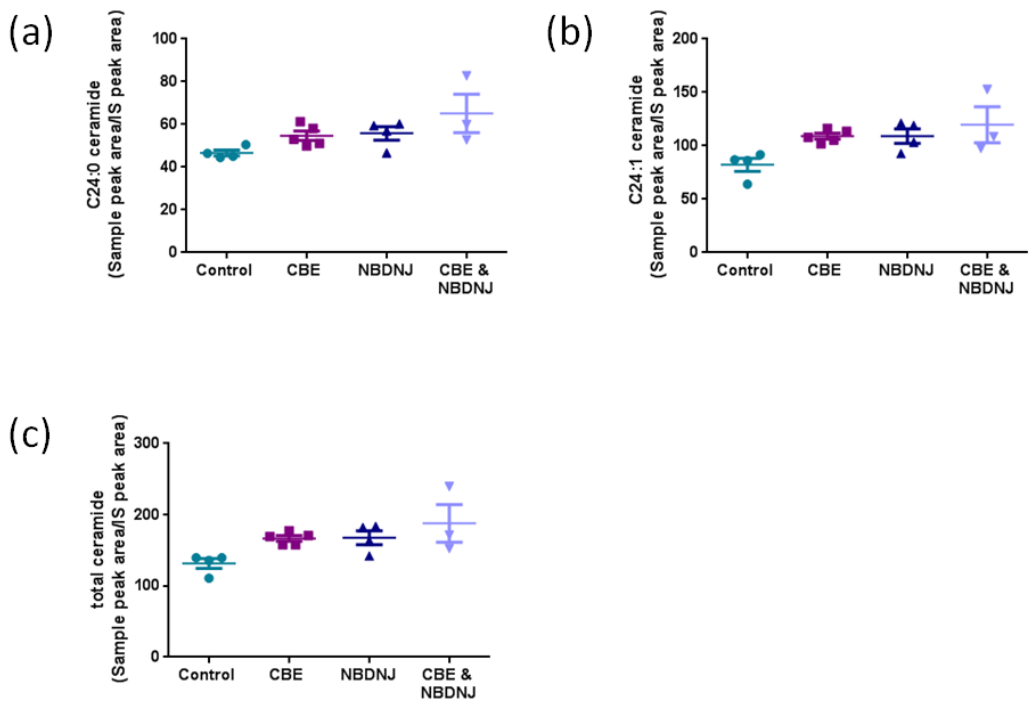

Figure 7.6 - Results of (a) C24:0, (b) C24:1 and (c) total measured ceramide analysis in SH-SY5Y cells.

Control = uninhibited cells, CBE = GBA1 inhibited cells, NBDNJ = GBA2 inhibited cells and CBE & NBDNJ = GBA1 and GBA2 inhibited cells. Values displayed are the peak area of the ceramide of interest divided by the peak area of the internal standard for that sample.

7.4 CONCLUSION

In this chapter a (UP)LC-MS/MS method for the analysis of ceramide isoforms has been described. Whilst clearly able to identify the individual species, further work is required to fully validate the method, i.e. to permit full quantification of the ceramide species of interest. However, the approach applied here has permitted the evaluation of the relative amounts of various ceramide isoforms in SH-SY5Y cells as displayed in **Figure 7.7**.

C24:0 and C24:1 ceramides are the predominant isoforms present in SH-SY5Y cells. Inhibition of GBA1 and/or GBA2 had no significant effect on the levels of any of the measureable ceramide isoforms. C24:0 and C24:1 are known to predominate in CNS and both have been shown to be pro-proliferative and have roles in autophagy and membrane fluidity [295].

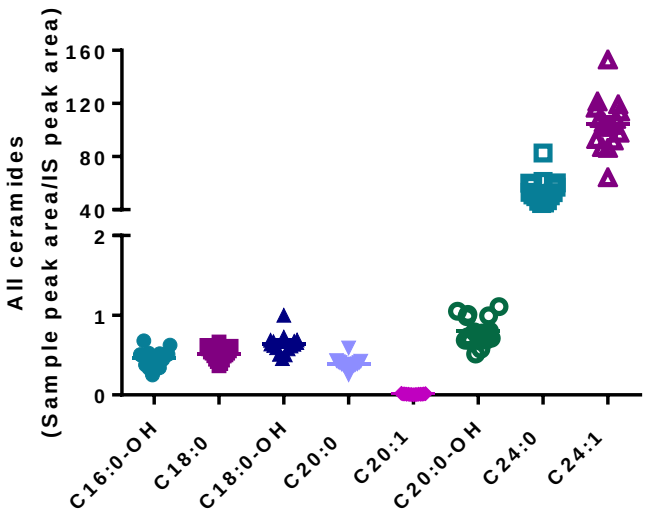

Figure 7.7- The comparative levels of the ceramides detected in SH-SY5Y cells.

The data includes all samples assayed i.e. GBA1 1 and/or GBA2 inhibited and uninhibited cells. C24 ceramides are the predominant isoform detected.

Inhibition of neuronal GBA1, GBA2 or both had no statistically significant effect on the relative abundance of the ceramide isoforms studied. However, the apparent trend for the relative amounts of some of the ceramides to increase in response to GBA1/GBA2 inhibition warrants further investigation. Further refinement of the method with regards to quantification of the individual isoforms and increasing the sample size should allow more detailed evaluation of this apparent trend. Alternatively, In view of the number of metabolic pathways that are known to be involved in ceramide as illustrated in **Figure 7.1**, it is possible that inhibition of one enzyme, i.e. GBA1, results in an increase in flux through another enzyme thereby maintaining ceramide homeostasis. Whilst it remains to be documented as to whether perturbation of ceramide metabolism plays a pivotal role in the pathogenesis of GBA1 deficient states, consideration of the role of the accumulating substrate, GlcCer also deserves attention. With this in mind, evaluation of the GlcCer to ceramide ratio has been suggested to be informative with regards to evaluating disease severity and treatment responsiveness in GD [301]. Further work will therefore include quantification of this GBA1 (and GBA2) substrate.

Chapter 8 General discussion

8.1 Discussion

Gaucher disease is the most common of the LSDs with an overall incidence of approximately 1:40,000 individuals, but it is much more common amongst individuals of Ashkenazi Jewish origin [29]. It is usually delineated into three types with the majority of patients having the "non-neuropathic" type I disease with fewer patients having the neuronopathic type II and type III forms [22]. However, neurological disease has been diagnosed in GD type I patients but with different signs, symptoms and severity compared to those associated with types II and III GD disease [31]. GD is an autosomal recessive disorder caused by mutations in the GBA1 gene located on the long arm of chromosome 1 (1q21). So far, more than 400 mutations have been described in the GBA1 gene. [35]. Mutations lead to low or deficient levels of the lysosomal enzyme GBA1 (β-glucocerebrosidase/β-glucosidase/β–glucosylceramidase (EC 3.2.1.45)). GBA1 is responsible for the penultimate step of the lysosomal degradation of glycosphingolipids i.e. the degradation of glucosylceramide to ceramide and glucose. The ceramide is further degraded by ceramidase to sphingosine and fatty acid. An alternative substrate, glucosylsphingosine, is also degraded by GBA1 into glucose and sphingosine [23]. GD displays vast phenotypic variations among patients including those with the same genotype and the discordant GD phenotypes observed in some monozygotic twins demonstrate the complexity of the disorder and supports a role for genetic modifiers [23, 90-93, 95-97].

Although GD was first described in 1882 [22] and Parkinson's disease in 1817 [138] it was only in 1996 that it was recognised that there was a relationship between the two [153]. GBA mutations are found in 5–10% of PD patients, making them numerically the most important risk factor for the disease identified to date [165] and GBA1 mutations are found at a higher frequency than any other known Parkinson's disease gene in the UK [166]. However, the phenotypic variations observed in GD also applies to the situation where, although they have a significantly higher risk of doing so than the general population, the majority of GD

patients and carriers do not develop Parkinson's disease [87, 170]. This therefore raises the possibility that other disease modifying factors, such as GBA2 activity, influence the ultimate clinical picture.

The initial aim of this thesis was to establish enzyme assays for GBA1 and GBA2 and to record relative activities in a range of tissue types. Subsequently these assays were used to document enzyme activities in GD and PD patient samples and a mouse model of GD. Building on the findings, cellular models (GBA1/GBA2 inhibition, oxidative stress, mitochondrial dysfunction) pertinent to studying GD/PD were used to further study the interplay between GBA1 and GBA2. To begin to further evaluate the effects on enzyme expression, western blotting was performed whilst mass spectrometry was utilised to generate data relating to the downstream effects on ceramide levels of GBA1 or GBA2 inhibition.

GBA1 and GBA2 Activities

Maximal GBA1 activity was determined here by performing incubations with sodium taurocholate. This well established approach is used in highly specialised diagnostic and research laboratories and exploits the fact that sodium taurocholate activates GBA1 and inhibits GBA2 [213]. For assessment of GBA2, NBDNJ was used as specific inhibitor of GBA2 [228]. Initially CBE was considered as an inhibitor of GBA1, thereby leaving only GBA2 activity. However, it was found that it also had the capacity at high doses to inhibit GBA2, an observation that has now also been reported by others [113, 228]. This finding means that careful use of CBE is required to ensure only inhibition of GBA1 occurs in experimental situations. However, NBDNJ permitted calculation of GBA2 activity by performing two incubations, i.e. one in the absence of NBDNJ (total GBA activity) and one in the presence (activity minus GBA2); the difference therefore representing GBA2 activity.

Using this approach, marked tissue differences in GBA1 and GBA2 activities and their relative proportions were found. GBA1 activity in fibroblasts was almost exclusively GBA1 whereas in brain GBA2 accounted for the majority of GBA activity. This predominance of GBA2 identified is supported by other studies that report

markedly increased gene expression of GBA2 in brain when compared to other tissue [112]. Taken together these findings may point to a particular role for GBA2 within the CNS. Expanding the study to cultured neural cells revealed that GBA1 and GBA2 activities were greater in the neuronal (SH-SY5Y) cell line when compared to the astrocytoma (1321N1) cells. Such data might point to the suggestion that within the brain, it is the neuronal cells that account for the majority of detectable GBA activity. However, further studies are now clearly required, including the use of primary cultures, to substantiate this suggestion.

GBA1 in PD and dystonia

Loss of brain GBA1 (activity and protein) has been reported in the post mortem brain of patients with idiopathic PD [192]. Since no GBA1 mutations could be detected, this raises the suggestion that the disease process itself might be responsible for the loss of enzyme. To build on this observation, leucocyte GBA1 activity was assessed here in patients with a diagnosis of PD or dystonia. Almost 13% of patients tested were found to have activities below the reference range. None of these patients were found to have GBA1 mutations. Furthermore, neither L-dopa nor its major metabolite, 3-methyl-dopa were found to influence GBA1 activity. Likewise, there was no evidence of an age-related decrease in GBA1 activities. Taken together, these findings again point to the disease process somehow influencing GBA1 activity. The finding of low activity peripheral to the brain might point to a mechanism, in a subset of patients, which, if identified, could provide further insight into the pathogenic mechanisms responsible for PD and dystonia. The findings reported in this study in leucocytes is substantiated by a finding, using dried blood spots, that also reports decreased GBA1 activity in iPD patients [246].

GBA1 and GBA2 Interactions

Determination of GBA2 in GD leucocytes revealed a wide spectrum of activities. Further inspection revealed two distinct groups. Thus, 54% of newly diagnosed GD patients had activities that were markedly elevated, the reminder having activities comparable to the control group. To evaluate these observations further, GBA2

activity was assessed in the brain of a GBA1 deficient mouse model and was found to be significantly elevated. This increase in activity is also consistent with a report of a strong increase of GBA2 protein and mRNA expression in GBA1 deficient mice fibroblasts [310].

Whilst there appears to be interplay between GBA1 and GBA2 in GBA1 knockout mice, GD patients appear to be a more heterogeneous group. Clearly, further work now has to be undertaken as to whether GBA2 status is in any way correlated with GD disease severity and the risk of developing PD.

How then might alterations in GBA2 influence the clinical picture when GBA1 activity is compromised? GBA2 is a non-lysosomal beta-glucosidase capable of hydrolysing the same substrates as GBA1 [134]. Mutations in GBA2 have been identified in patients with ataxia [122, 123], hereditary spastic paraplegia [120, 121] and Marinesco-Sjögren syndrome [116]. While a number of disease states have now been attributed to mutations in GBA2, the biochemical roles for GBA2 are yet to be fully defined. It has been proposed that ceramide formed via GBA1 is degraded to sphingosine and that generated by GBA2 utilised for sphingomyelin formation [108]. However, in view of the overlapping substrate specificity of GBA1 and GBA2, it is possible that GBA2 may contribute to glucosylceramide clearance as illustrated in **Figure 8.1** and therefore, GBA2 could be a modifying factor in GD and also in PD-GBA1. Under normal conditions GlcCer is metabolised in the lysosome by GBA1 and extra-lysosomally by GBA2 as shown in **Figure 8.1(a)**. In GD (**Figure 8.1 b)** the accumulating GlcCer could be metabolised outside the lysosome which could lead to excessive or unwanted ceramide levels associated with increased GBA2 activity or protein expression. In GD patients with naturally low or pharmaceutically inhibited GBA2 **(Figure 8.1 C)**, GlcCer would accumulate not just in the lysosome but also in the cytosol. This would lead to even greater GlcCer levels and could potentially lead to lower than normal or required levels of ceramide. Supporting this, it has been reported that GBA2 knockout mice exhibit accumulation of brain glucosylceramide [109]. Concerning disease pathogenesis and the influence of GBA2, GBA2 gene deletion has been reported to rescue the clinical phenotype in a GD mouse model [225]. However, the effects on the CNS, where GBA2

predominates, were not considered. Concerning this point, it is of note that one of the GD/PD patients studied here had undetectable GBA2 in his leucocytes (section 4.4.2). His clinical phenotype was of mild GD, consistent with the suggestion that loss of GBA2 activity may be beneficial in dictating GD severity [193]. However, he had marked early onset Parkinsonism. This is only one case, but given the predominance of GBA2 in brain, further work is perhaps required to ascertain whether loss of GBA2 has a differential effect in the periphery when compared to the CNS.

To study the interplay between GBA1 and GBA2 further, SH-SY5Y cells were used. Blockade of GBA1 was not found to be associated with an increase in GBA2 activity or protein expression. Similarly, inhibition of GBA2 was not found to be associated with changes in GBA1. Whilst these findings may not immediately lend support to a strong interaction between these two enzyme systems, this was a relatively acute study and hence factors such as significant substrate accumulation may not have occurred. Whilst substrate (GlcCer) was not studied here, levels of the product (ceramide) were assessed. Despite not being a fully quantitative study, relative comparisons do not point to any significant differences following inhibition of GBA1, GBA2 or both. This finding, if confirmed, could reflect the relatively acute nature of the study or point to involvement of alternative pathways to ensure ceramide levels are maintained.

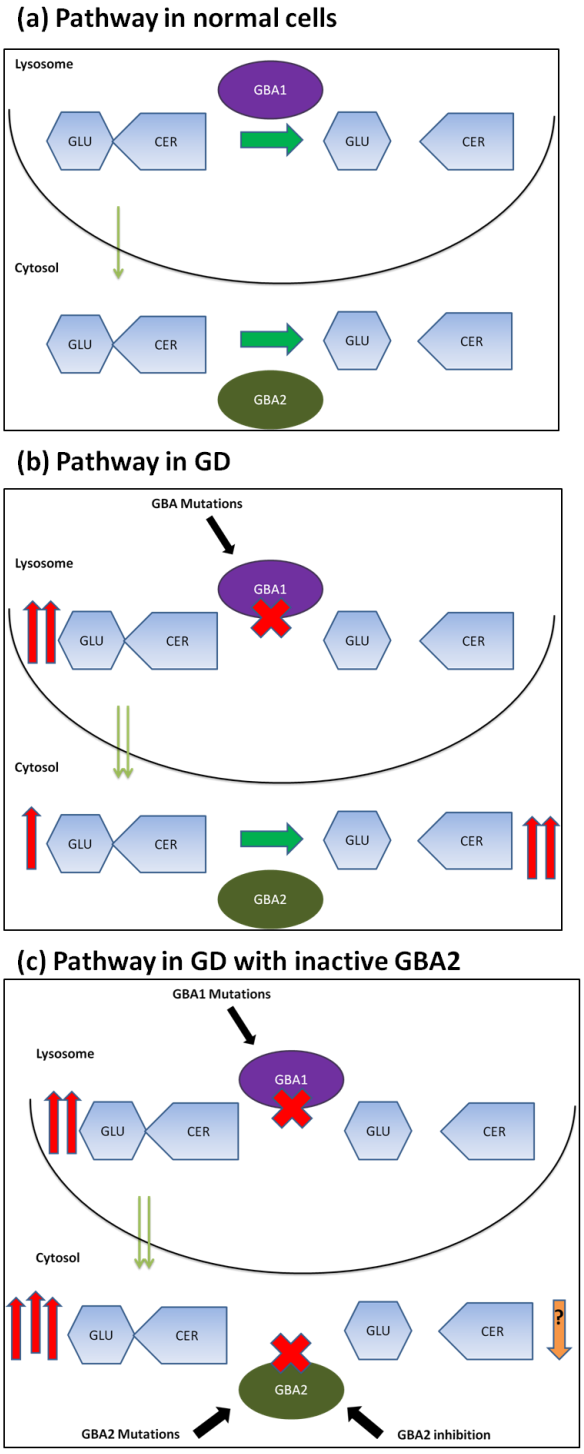

Figure 8.1 - Different potential fates for GlcCer and its metabolite ceramide.

(a) In unaffected cells GlcCer is metabolised in the lysosome by GBA 1 and the cytosol by GBA2. (b) In GD, GlcCer accumulates in the lysosome and is metabolised in the cytosol by GBA2 potentially leading to high levels of ceramides. (c) In GD cells, where GBA2 is naturally low or inhibited, GlcCer continues to accumulate.

Oxidative stress and Mitochondrial Function

Oxidative stress and loss of mitochondrial function, particularly at the level of complex I, is associated with PD [252, 311]. Since, oxidative stress has been shown to modify and inhibit some enzymes [312, 313], and energy is required for protein (enzyme) synthesis and processing, cellular models of oxidative stress or complex I deficiency were created, i.e. with the aim of documenting effects upon GBA1 and GBA2 activities. Whilst oxidative stress was associated with loss in cell viability, there were no apparent effects upon GBA1 or GBA2 activities. Similarly inhibition of complex I was not associated with any effect upon GBA1 or GBA2 activities. Thus, these enzymes appear relatively robust, at least under the conditions employed, and this may point away from oxidative stress, or loss of mitochondrial function, being responsible for the decrease of GBA1 associated with idiopathic PD. Intriguingly, another lysosomal enzyme, total beta-hexosaminidase, was found to be significantly increased in activity in the neuronal cells exposed to H_20_2. Whilst the mechanism for this apparent up-regulation in activity is not known, It is of interest to note that total beta-hexosaminidase has been found to be significantly increased in CSF from PD patients [276]. Furthermore, it is has been proposed that this enzyme might be biomarker for PD [278].

The findings also reported here that inhibition of GBA1, GBA2 or both enzymes does not influence cellular susceptibility to oxidative stress or mitochondrial content suggests that these enzymes may not directly contribute to these phenomena in PD.

8.2 Conclusion

Data presented here points to a particular role for GBA2 in the brain and suggests an interplay between this enzyme and GBA1. This interaction appears variable in GD patients and raises the possibility that GBA2 activity could be a factor with regards to clinical phenotype.

When considering idiopathic PD or dystonia, loss of GBA1 activity can be demonstrated in the leucocytes of some patients. These relatively accessible cells

could therefore be useful for future studies into disease mechanisms and for monitoring treatment efficacy.

Loss of mitochondrial function or oxidative stress may not be a contributing factor to the loss of GBA1 activity associated with idiopathic PD.

Inhibition of GBA1 or GBA2 activity does not appear to markedly influence ceramide status raising the possibility that alternative pathways are utilised to maintain cellular levels.

8.3 Further Work

Use differentiated SH-SY5Y cells and primary neuronal cultures to further evaluate the interaction between GBA1 and GBA2. In addition, to account for glial-neuronal interactions, establish a co-culture system of astrocytes and neurons. Use this system to consider in more detail the interplay between GBA1 and GBA2.

Use of gene silencing techniques to further evaluate the effects of loss of GBA1 and/or GBA2 activity in both neuronal and glial cells.

Apply western blotting of GBA1 and GBA2 to GD/PD patient leucocytes and relate to enzyme activities.

To fully develop a mass spectrometry method for the quantification of GlcCer and ceramides. Use this methodology to document GlcCer and ceramides in leucocytes, plasma and, where possible, CSF of patients with GD and PD.

Evaluate further the potential of total b-hexosaminidase to act as a biomarker for PD. Activity of this enzyme to be determined in CSF, plasma and leucocytes from PD patients in addition in other neurodegenerative conditions and disease states associated with oxidative stress, e.g. involving immune response activation.

Identify the effects in more detail of inhibition of GBA1 (+/- GBA2) on cellular dopamine metabolism. Dopamine availability is ultimately compromised in PD and consequently identification of mechanistic link between lysosomal metabolism and dopamine could provide new insights into disease pathogenesis and novel

therapeutic targets. Preliminary collaborative work has so far identified that in CBE treated SH-SY5Y cells, increased breakdown of dopamine may occur [314].

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Publications

Papers

Alonso-Canovas, A., Katschnig, P., Tucci, A., Carecchio, M., Wood, N.W., Edwards, M., Martinez Castrillo, J.C., **Burke, D**., Heales, S., and Bhatia, K.P. (2010) **Atypical parkinsonism with apraxia and supranuclear gaze abnormalities in type 1 Gaucher disease**. Expanding the spectrum: case report and literature review. Mov Disord 25, 1506-1509

Burke DG, Rahim AA, Waddington SN, Karlsson S, Enquist I, Bhatia K, et al. **Increased glucocerebrosidase (GBA) 2 activity in GBA1 deficient mice brains and in Gaucher leucocytes**. J Inherit Metab Dis. 2013 Sep;36(5):869-72.

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Keatinge, M., Bui, H., Menke, A., Chen, Y.C., Sokol, A.M., Bai, Q., Ellett, F., Da Costa, M., **Burke, D.,** Gegg, M., Trollope, L., Payne, T., McTighe, A., Mortiboys, H., de Jager, S., Nuthall, H., Kuo, M.S., Fleming, A., Schapira, A.H., Renshaw, S.A., Highley, J.R., Chacinska, A., Panula, P., Burton, E.A., O'Neill, M.J., and Bandmann, O. **Glucocerebrosidase 1 deficient Danio rerio mirror key pathological aspects of human Gaucher disease and provide evidence of early microglial activation preceding alpha-synuclein-independent neuronal cell death.** Hum Mol Genet. 2015 Dec 1;24(23):6640-52.

de la Fuente C, **Burke DG**, Eaton S, Heales SJ. **Inhibition of neuronal mitochondrial complex I or lysosomal glucocerebrosidase is associated with increased dopamine and serotonin turnover.** Neurochem Int. 2017 Feb 24.

Platform presentations

GBA 1 and 2 activity in brain, and cultured neuronal and astrocyte cell lines. 5th Neuronopathic Gaucher disease family conference. Reading, UK. April 30th 2011.

GBA 1 and 2- factors to consider in Parkinson's disease. 18th ESGLD (European Study Group on Lysosomal Diseases) conference, Helsinki, Finland. September 5th 2011.

Glucocerebrosidase 1 and 2 - factors to consider in the pathogenesis of Parkinson's disease. 8th annual WORLD (We're organising research on lysosomal disease) symposium, San Diego, USA. February 8th 2012.

Lysosomal β-glucosidase (GBA1) and non-lysosomal β-glucosidase (GBA2), potential involvement in the pathogenesis of Gaucher disease/Parkinson disease. 11th Annual WORLD symposium. Orlando, Florida, USA. February 10th 2015.

Brain GBA1 & 2 activities: Factors to consider in PD. The 12th International Conference on Alzheimer's and Parkinson's diseases (AD/PD 2015) Nice, France. March 18th 2015.

Studying the link between Gaucher and Parkinson's disease; cell models and disease modifying factors. 12th EWGGD (European working group on Gaucher disease) congress. Zaragoza, Spain. June 29th 2016.

Poster presentations

Glucocerebrosidase activity and Parkinsonism, Potential pathogenic mechanisms. 2nd Parkinson's UK Research Conference. York, UK. November 1st 2010.

Glucocerebrosidase activities- factors to consider in the pathogenesis of Parkinson's disease". Gaucher Leadership forum, Budapest, Hungary. September 22nd 2011. Poster won first prize in poster competition.

The role of enzymology in determining Gaucher status in Parkinson's disease. 10th International EWGGD Meeting, Paris, France. June 28th 2012.

Glucocerebrosidase activities and Gaucher disease, potential neuronal glial differences. 19TH ESGLD workshop. Leibnitz, Austria. September 25th 2013.

GBA1 and GBA2, factors to consider in Parkinson's disease**.** 11th International meeting of the EWGGD. Haifa, Israel. June 25th 2014.